

Britain's new machinery for research

First news of the British Government's new policy for supporting science suggest that some of the proposed changes may be beneficial. But the new policy must be judged in the round, which is not yet possible.

MR William Waldegrave, the British Cabinet minister with responsibility for the year-old Office of Science and Technology, has been moving the furniture energetically, but to what effect? Mr William Waldegrave's white paper (policy statement) due to be published this week does, as expected, give Britain new machinery for spending money on civil science. There is nothing wrong with that. Obviously there is a danger that well established institutions, even when they are run by scientists on behalf of science, may become over-used to established practices and procedures. A shake-up from time to time is no bad thing. But the test of whether this latest shake-up will be beneficial can be judged from the full text of the policy proposed and from the way in which it is implemented. That must come later.

Meanwhile, particle physicists and astronomers in Britain will be relieved. By segregating their dependence on public funds from that of the rest of basic science, the government proposes to institutionalise their existence. For some years at least, Britain's continued membership of the European High-Energy Physics Laboratory (CERN) at Geneva should be free from scrutiny. That, at least in the short term, should dispense with the frequent re-examination of Britain's continued membership of CERN. The obvious danger is that the creation of the Astronomy and Particle Physics Research Council may ensure funds for accelerator physics long after the present need has been replaced by others.

The redrawing of the responsibilities of the remaining research councils is reflected in their new names. With astronomy and particle physics hived off, the research council named for "science and engineering" becomes that for "engineering and physical science", while "food and agricultural" becomes "biotechnology and biological". But it seems that the precise demarkation between these new councils has not yet been settled. Yet that is the process in which blood can easily be spilled. It will be tempting to shuffle important research institutes about as if they were mere pawns on a chessboard, putting them where they appear best to belong to the newly designated research councils. So much damage has been done to the morale of British science by needless shuffling in the past decade and a half that those concerned had better be careful.

Meanwhile, it is plain that the creation of an extra research council is partly offset by the proposal to create the new post of director-general (with oversight of the research councils) in the Office of Science and Technology. This

centralizing step has been forced on the government by logic. Having (for good reasons) decided to abolish the Advisory Board for the Research Councils, and to take on its own shoulders responsibility for sharing out funds among its corporate dependants, there has to be a person who can do the job for long enough to learn how, and then to be effective. At least at the beginning, it will be a thankless task. Researchers will be complaining that too much change is expected of them, the government and others will want to know where is all the wealth the new arrangements are supposed to create. That is the most radical (because it is the most demanding) of all the moves that Waldegrave proposes on the British government's behalf.

For the rest, this journal will reserve its opinion on the white paper until the arguments for the proposed changes, and the language in which they are described, can be read as a connected piece. Except, that is, on one point. It seems not yet to have been decided whether the new research council and those which have been renamed will continue to be responsible for supporting research students and for paying pittances to them every month. Yet this is an area in which British science has become far too complacent. Research studentships are allocated to university departments judged competent in research, which (within broad guidelines) are allowed to fill them as they choose. Especially if the government hopes to formalise entry into courses leading to research degrees, there is every reason why the selection of graduate students and their projects should be handled separately from the administration of research funds. Ideally, it should be for students to choose where and with whom they work. Certainly graduate students should not be regarded as the laboratory fodder they have too often been in the past. □

Delaney must go

The US Congress should rewrite the food and drug law that bans chemicals that may be quite harmless.

POTATOES, tomatoes, grapes and mint. These and other foods that grow best with the help of agricultural pesticides will have to take their chances with nature because the US Environmental Protection Agency (EPA) has had to deny farmers and pesticide manufacturers permission to use



more than 20 chemicals even though the agency believes they are perfectly safe. The EPA, often criticized as an agency strong on politics but weak on good science, has been struggling to bring rationality to its way of making decisions on pesticides, fertilizers and other agricultural products whose use could leave residues in food. Now it has been kept from good sense by an arcane and outdated law.

Arguing that there are data to show that the dangers of many agents, in particular chlordane and bifenthrin (which confer pest resistance on tomatoes), are negligible, the EPA was summoning up its courage to grant exemptions to manufacturers from the infamous Delaney clause. That is the requirement of the food and drug law that sets a 'zero tolerance' standard for agricultural chemicals for which there is evidence of carcinogenicity of any kind. Written in 1958, when techniques to test for chemical residues in food were only crude, the law now precludes the use of compounds that can be detected in concentrations as low as a few parts in a billion. The tyranny of Delaney over good sense marches in step with the improvement of analytical sensitivity.

Yet EPA's modest proposal is simply to evaluate potentially hazardous chemicals according to a standard of 'negligible risk', rather than the Delaney standard, which demands no detectable residue at all. Sadly, the US Supreme Court ruled in February that EPA must comply with the letter of the law, meaning that the agency has no choice but to make decisions that cannot be justified by science. The only solution is to change the law, and that is what the administration and Congress should do. EPA Administrator Carol M. Browner has made it known that she would support new legislation based on contemporary science and she should be encouraged to pursue that goal.

Indeed, the case is now stronger than ever. During the past few years, there has been a good deal of research aimed at establishing a scientific basis for new standards. The National Institute for Environmental Health Sciences (part of the National Institutes of Health) has supported a series of 'predictive' studies of potential carcinogens by exposing rodents (in separate experiments) to 44 agents for periods as long as two years. Using information such as gross chemical structure, known mutagenicity data, interaction with cell receptors and other indicators of chemical behaviour, the object has been to produce a sound base for predicting which chemicals are likely to be carcinogenic in trace amounts and which are less likely to pose a threat. The EPA, no doubt, will pay attention to the results of these studies, which are even now being readied for publication.

Congress and the administration should pay attention as well. The United States, like other nations, cannot afford to ban useful products just to satisfy the unsubstantiated fears of committed environmentalists whose heart is in the right place but whose judgement sometimes ignores what scientific understanding has to offer. It is high time that the law was brought into compliance with scientific reality. □

Academic inequality

Oxford women have won an in proving that universities have a responsibility to foster their promotion.

As the success of attempts in the past few decades to end bias against women in universities is assessed, it is becoming clear that an equal opportunities policy can work only if the organization concerned really wants it to. If top universities truly want to be seen to lead the field in sex equality — as indeed perhaps is their responsibility — their policies will have to be backed up by action, and soon.

At a meeting of the University of Oxford's academic council, or "Congregation" last week, equality-minded members caused a stir by blocking the university's plans to create 15 new professorships — all of which would probably have been occupied by men. The rebels said they would rather the money was spent on establishing additional 'readerships' — one step down from professorships — to be filled by promoted lecturers. They believe that, because most women on Oxford's faculty are lecturers, they would stand a better chance of promotion if more readerships were available.

Although women make up 14.1 per cent of Oxford's academic staff, they account for only 3.1 per cent of professors, some way below the British average of 4.4 per cent. In a cruel irony, no woman has been awarded a new professorship since the university adopted its equal opportunities policy at the end of the last decade.

For what it is worth, the picture is just as grim at Harvard University, where the discrepancy between stated intentions and practice is even more glaring. The university claimed to be initiating a programme of affirmative action as long ago as 1970, but a survey by the Ad Hoc Committee on Gender Equality at Harvard Radcliffe reveals that, since then, the number of women on the faculty has actually declined, while the percentage of tenured women has climbed imperceptibly to a pitifully low 8.2 per cent. According to Peggy Schwartzler, chairwoman of the committee, the university's governing body has been largely unresponsive to calls for it to meet its pledges. "Without more vigorous support from our leadership", says Schwartzler, "we can no longer continue to press on toward our goal of full gender equality at Harvard Radcliffe."

Youthful optimism has long since deserted organisations such as Schwartzler's, and with it has vanished the promise of an early or speedy end to the purgatory of women bent on scaling the academic heights. 'Quota' systems, for their part, have come and gone with little effect but to exacerbate the controversy over 'political correctness'. The next step towards anything more than tokenism is hard to make out. Change will come only when the universities genuinely want it to happen. If educational organizations dedicated to the preservation of ideas in our culture refuse to take sex equality seriously, when will be it be realistic for anybody else to do so? □

Waldegrave wants to carve up SERC and create stronger political links

London. The British government is proposing to break down what officials describe as a "Chinese wall" between science and the political process by appointing a director-general in the government's Office of Science and Technology (OST) to take direct responsibility for the work of the research councils.

In doing so, William Waldegrave, Chancellor of the Duchy of Lancaster and the cabinet minister responsible for science, is seeking to reverse a tradition of protecting the administration of science from direct political influence. The tradition began with the creation in 1920 of the Medical Research Council — and the formulation of the so-called 'Haldane Principle' — but has now left Britain out of step with most of its economic competitors.

The government is also planning to carve up the responsibilities of the Science and Engineering Research Council (SERC) and to create two new councils, one for particle physics and the other for engineering and physical sciences. In addition, SERC's biological research will be merged into the Agricultural and Food Research Council to form a single Biotechnology and Biology Research Council.

The creation of a director-general for research councils and the splitting up of the SERC (the other four councils will remain intact) are the most radical changes to emerge from the government's White Paper (policy document) on science, which was published this week. The White Paper will disappoint those who had been hoping for a major increase in research funds as well as those wanting a strong, centralized science policy; individual government departments will retain most of their existing responsibilities, and there will not be large amounts of money for new initiatives.

In contrast, Waldegrave is seeking to achieve the twin goals of boosting wealth creation and the quality of life by bringing the world of science and engineering decision-making into the political process to ensure that this process is "infested" with both activities. At the same time, all the research councils will be given an explicit mission of working towards these twin goals.

Since the early 1970s, the work of the research councils has been coordinated by the Advisory Board for the Research Councils (ABRC), which in particular has advised the government on the distribution of funds between the councils. But the creation last year of OST has made the role of this body ambiguous.

The government has now decided to abolish it, and to absorb executive responsibility directly into the OST. The new director-general for research councils will be responsible to the permanent secretary of the OST for the way that individual research councils are run and for ensuring collaboration between them.

The abolition of the ABRC — and at the same time of the Advisory Council on Sci-

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Waldegrave wants research to be part of politics.

ence and Technology — will mean a general shake-up in the structure of advisory committees. The main lines of government policy will now be discussed by a new high-level Council on Science and Technology, which will be chaired by the minister for science on behalf of the prime minister, and whose members will include representatives of industry, government and the academic world.

The Council will have a secretariat provided by the OST and will be headed by the government's chief scientist, Bill Mitchell. In addition, the new director-general for research councils will also have his or her own advisory group of outside experts.

The OST will not, as some had originally proposed, take over the research budgets of any of the individual government departments. Attempts to do this have been strongly resisted by the departments themselves, and the government now appears to believe that it would be a mistake to centralize the direction of departmental policies.

However the OST will keep a close eye on what departments are doing with their science budgets. Each department will be required to publish research spending plans, which will be compiled into a single document published annually as a five-year "forward look".

This exercise will allow the OST — which will also carry out a series of "technology foresight" exercises to develop agreement on long-term research needs — to make

suggestions to departments about what research they should or should not be carrying out to achieve these needs. Waldegrave himself sits on a leading cabinet spending committee, allowing him to keep departmental research spending plans under close review.

The splitting of SERC will mean that those activities considered to contribute towards the government's goal of wealth creation (including, for example, the new Innovative Manufacturing Initiative; see page 295) will come under the responsibility of the planned Engineering and Physical Sciences Research Council. The creation of a single Biotechnology and Biological Research Council is in line with proposals made several years ago. Giving particle physics its own research council will mean that additional costs incurred by swings in international exchange rates will now be jointly absorbed by all the research councils, and not just the SERC.

Waldegrave has decided to remove the uncertainty that has recently been hanging over university scientists by allowing the Department of Education to keep responsibility for funding the basic platform of university research through the three higher education funding councils. In addition to maintaining the dual support system in this way, the government also intends to improve career prospects for university scientists by introducing an extra year of study, leading to an MSc, for all graduates before starting postgraduate research. This period will be used to assess the suitability of an individual for a research career.

In addition, OST is considering the introduction of longer-term doctoral fellowships. And both universities and other institutions receiving government research funds will be required to demonstrate that they are taking steps to exercise greater concern for the career development of their research staff.

Finally, the government has decided to focus its efforts to stimulate public understanding of science on Britain's secondary schools. A fund will be set up to subsidize such efforts, organized on a regional basis, to which private bodies will also be asked to contribute.

In addition, a series of activities is planned leading to a major event in 2001, which marks the 150th anniversary of the Great Exhibition in London. But Waldegrave is clearly hoping to do better than Prince Albert in injecting science and technology into the political culture of contemporary Britain.

David Dickson

Japanese universities are slow to welcome foreign faculty

Tokyo. Members of Japan's ruling Liberal Democratic Party (LDP) have joined a chorus of voices calling for a dramatic increase in the number of foreign faculty members in national universities. An LDP project team led by Noriaki Watake submitted its recommendations earlier this month to two LDP committees on academic and cultural affairs, and last week a committee of academics that advise the Ministry of Education, Science and Culture (MESC) made a similar recommendation.

Despite this growing pressure, Japan's universities will probably be slow to change their employment practices. Although universities have since 1982 been allowed to hire non-Japanese professors, associate professors and lecturers, the number of such foreign faculty today stands at only 201, or 0.5 per cent of the approximately 37,000 faculty in Japan's 98 national universities.

Of these foreigners, only 17 enjoy permanent employment (tenure) like their Japanese colleagues (see table). The rest are on

temporary appointments, which are limited to a specified number of years (with the possibility of extension if their performance is good).

Furthermore, almost half of those with tenure have strong personal links to Japan. Eight are graduates of Japanese universities, two of the Koreans with tenure were born and educated in Japan and two other faculty members are of Japanese ancestry. Only four Western academics have tenure, and for two of them their speciality is teaching their native language.

The release of the LDP recommendations was followed a week later by a report from a committee of the University Council, a body of senior academics that advises MESC. Calling for more open employment practices in Japan's universities, the report says that more foreign faculty should be hired and that the terms of their employment should be adapted to individual needs.

The LDP and University Council recommendations echo the views of a team of

foreign and Japanese scientists who earlier this year proposed employment of more foreign faculty in an external review of the physics department of Tokyo University (see *Nature* **362**, 387; 1993). Among the national universities, Tokyo is the most progressive; it employs the largest number of foreign faculty, 13, and has given tenure to nearly half of them. Nevertheless, only a handful are scientists or engineers.

"In principle I agree completely with these [LDP] recommendations", says Akito Arima, former president of Tokyo University who retired from the university shortly after the external review was completed in March. "But in practice it will be very hard to do. Not so many foreign scientists want to stay in Japan. And there are many problems such as lack of international schools for their children and lack of suitable accommodation". Other leading Japanese academics say that institutions are wary of hiring foreign faculty because it is difficult for them to participate in meetings and join committees if they cannot speak and write Japanese.

"The language problem is real", says Robert Geller, a US geophysicist at Tokyo University and one of the few foreign faculty with tenure. "But it should not be used as an excuse. Foreigners new to Japan can make significant contributions to committees dealing with international issues and they can help in the production of English reports." Geller, who has lived in Japan for several years and who speaks and writes Japanese, has served on several faculty committees.

Geller says that the dearth of foreigners is closely related to the "lack of Japanese from other universities" on any faculty. Departments typically choose new faculty members from among their own students and there is very little movement between universities. For example, in Tokyo University's faculty of engineering, 99 per cent (998) of the 1,010 faculty members are graduates of Tokyo University. Such inbreeding needs to be eliminated before a significant influx of foreigners can be achieved, says Geller. Another obstacle is the small number of women faculty members in Japanese universities.

Although individual faculty members play an important role in bringing about change, there is also a need for the large number of universities that do not offer tenure to revise their internal regulations to allow employment of foreigners on equal terms. MESC, which administers the universities, must also provide strong leadership, an ingredient that so far seems lacking. Commenting before the release of the University Council report, Yoshikazu Hasegawa, director of the ministry's science and international affairs bureau, said about the LDP recommendations that "we will treat the suggestions of the legislature with respect, but we will not necessarily say yes to everything".

David Swinbanks

Foreigners with tenure in Japan

Tokyo University	Department	Rank	Graduate school
Paul Chen (US)	Law & Politics	Professor	Taiwan University
Robert Geller (US)	Earth & Planetary Physics	Assoc. Professor	Caltech, US
Srikantha Herath (Sri Lanka)	Inst. of Industrial Science	Assoc. Professor	Tokyo University
Shik Shin (Korea)	Inst. for Solid State Physics	Assoc. Professor	Tokyo University
Toshihiro An (Korea)*	Nuclear Power, Engineering	Lecturer	Tokyo University
Ha-Won Song (Korea)	Civil Engineering	Lecturer	Yonsei University
Kyushu University			
Tsutomu Takahashi (US)	Earth & Planetary Sciences	Professor	Hokkaido University
Michel Wolfgang (Germany)	German, Inst. of Languages and Cultures	Assoc. Professor	Frankfurt University
Kanazawa University			
Ho-Hi Lee (Korea)	Liberal Arts, Chemistry	Assoc. Professor	Kyoto University
Sang Young Nam (Korea)	Liberal Arts, Korean	Assoc. Professor	Tokyo Metropolitan U.
Shizuoka University			
Michael A.P. Lloyd (UK)	Liberal Arts, English	Assoc. Professor	London University
Wonn Soh (Korea)*	Earth Science	Lecturer	Kyoto University
Fukushima University			
Harold Lewis (US)	Economics	Assoc. Professor	State U. of New York at Binghamton, US
Tohoku University			
Jong Won Lee (Korea)	Law	Assoc. Professor	Tokyo University
Hirosaki University			
Victor Carpenter (US)	Liberal Arts, English	Assoc. Professor	Macalester College, US
Hitotsubashi University			
N/A (China)**	Economics, Chinese	Professor	—
KEK (National Laboratory for High Energy Physics)			
Tokio Kenneth Ohsaka (Canada)	Physics	Assoc. Professor	Carleton University

* now an associate professor at Tokai University

now associate professor

** retired in April 1993

Source: Ministry of Education, Science and Culture and "All Japan University Staff Directory 1992" (Kojun-sha)

Gibbons: SSC should have been international

Washington. The lack of international funding for the US Superconducting Super Collider (SSC) is "a mistake that we should never make again", says presidential science and technology adviser John Gibbons, but the \$10-billion project will nevertheless continue.

Gibbons says that the funding prospects for the proton-proton accelerator being built in Texas are "a mess" even though the Clinton administration decided earlier this year to prolong construction for three years, giving itself more time to attract international contributors and to make sure that all technical questions have been answered. With a fifth of the money already committed, however, Gibbons says that "we're simply going to have to try to work our way through" any more problems that arise and, if necessary, "we're just going to have to pay for it ourselves".

Congress will shortly take up the president's budget request of \$640 million for the SSC in fiscal year 1994, which begins on 1 October. Gibbons admits that "it's going to be tough choice" in the context of efforts to reduce the \$300-billion federal deficit, but he says that he is no longer concerned about possible cost overruns and technical difficulties involved in manufacturing the 8,500 dipole magnets that will line the SSC's 54-mile-long tunnel.

Gibbons discussed with *Nature* last week the balance of federal research among agencies, the role of the national laboratories and the emerging structure of his 40-member staff at the Office of Science and Technology Policy (OSTP), which he directs. He also predicted that an announcement would be made "very soon" on the president's nominees to head the National Science Foundation (NSF) and the National Institutes of Health (NIH).

The Clinton administration has been criticized, in particular by biomedical researchers, for keeping science in the shadows of a widely publicized technology initiative and a supplemental appropriations package (since rejected by Congress) emphasizing the role of technology in creating jobs. Gibbons, a physicist who joined the administration in January after spending 13 years as director of the congressional Office of Technology Assessment, defends the administration's policies and asserts that "science is probably the most productive investment you can make".

Gibbons says that he is "very troubled" by the allocation of the \$73 billion that the government will spend this year on research and development, adding that the US investment in science and technology — with its 59:41 balance between military and civil-

ian research — "does not match our national goals". However, when asked whether basic-research agencies such as NSF and NIH should receive more money, Gibbons says "not necessarily", confessing that "I'm not sure what the magic number is for basic research".

One sector often mentioned as ripe for pruning are the 725 federal laboratories, in particular some of the national laboratories run by the Department of Energy (DOE).

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Gibbons wants many good men and women.

Although Gibbons says that he would be hard-pressed to defend the continued existence of one, much less two, nuclear weapons laboratories — currently at Los Alamos, New Mexico, and Livermore, California — he does not favour closing any of the DOE laboratories.

"I think that the notion of closing or opening is not as critical as what you do with those resources", he says. "You need to look at the nature of the resource, what kinds of people and equipment there are and the optimal way to use them. That's the issue facing DOE."

Gibbons places great importance on the number of cooperative research agreements between federal laboratories and industry and says that a laboratory's ability to attract 10–20 per cent of its budget from the private sector "poses a good market test" of the utility of its research programme. But he says that such a test "is only a start" and that additional information is needed before the fate of any laboratory is decided.

As director of OSTP, Gibbons is respon-

sible for coordinating federal spending on science and technology. He says that his job is made easier by the absence of "warring ideologies" like those that confounded the previous two administrations on such issues as industrial policy and global warming and that he hopes to build on the work of his predecessor, D. Allan Bromley, in creating interagency panels known as Federal Coordinating Committees for Science, Engineering and Technology (FCCSET).

These panels are involved in six research initiatives — covering global climate change, biotechnology, advanced manufacturing, science education, high-performance computing and materials — with a combined budget next year of \$12.5 billion (see *Nature* **362**, 776; 1993).

The White House has just begun a review of these initiatives as the first step in preparing the 1995 budget, and Gibbons says that it is likely that some, in particular advanced materials, may not be retained in their present form. To sharpen that analysis, a second group has been formed consisting of the deputy and assistant secretaries from each of the agencies funding research in one or more of the areas. This deputies' group, Gibbons says, provides "one-stop shopping" for those needing information about FCCSET-related efforts at each agency as well as a review of existing programmes.

Gibbons has played a leading role in finding an NSF director to succeed Walter Massey, who left two months ago to become provost of the University of California system. He admits to being "disappointed" when his first choice, astronomer Sandra Faber, decided to remain at the University of California at Santa Cruz, and says that next time "I'm going to make sure that the person is ready to say yes" before the post is offered. (Faber says that she was "very interested" in the NSF post but that two events later this year involving projects on which she has worked for a decade — the start of operation of the Keck telescope and the repair of the Hubble space telescope — led her to decline the offer.) But he is even more disturbed by the paucity of qualified applicants.

"If there is any shortage in science, it's of people able and willing to do jobs like this one, or at OTA, NSF or NIH", he says. "One of the problems is that the system doesn't provide many opportunities at lower levels to acquire the necessary skills." Although such jobs are not for everyone, he says that not enough senior researchers "feel an obligation to apply what they have learned and experienced" outside the laboratory.

Jeffrey Mervis

Budgetary pressures squeeze German research institutes

Munich. The Hahn-Meitner Institute in Berlin will abandon its nuclear physics and electronics research activities in one of the first comprehensive responses by a national research centre to demands from the German government to cut costs and increase accountability.

The 16 centres, most of them founded 30 years ago, carry out long-term research beyond the means of a university. In 1991, the former research minister, Heinz Riesenhuber, laid out plans to change a system that the government had long felt was over-staffed, inflexible and lacking in external evaluations. The general financial constraints that have followed reunification and the creation of three new national research centres in the former East Germany have focused attention on the centres, which together receive 70 per cent of the federal government's research budget.

Riesenhuber realigned priorities for the national research centres, which get 90 per cent of their money from the federal government and the rest from the *Länder*, by lowering federal support and asking them to reduce their work force of 22,000 by 1,800. But the cuts are not uniform. Areas such as nuclear energy research, politically unattractive in Europe's 'greenest' country, suffered badly while more fashionable areas such as climate research have been better protected. In addition, the operating budgets of most institutes have been frozen at 1992 levels until at least 1995.

The Hahn-Meitner Institute must lose

at least 50 people — 10 per cent of its permanent staff. Last month, the institute's advisory board decided to use the restructuring to concentrate on solar energy and structures research. Nuclear technology research will be completely wound down by 1995, after which its heavy-ion accelerator will be used only to treat patients with eye cancer.

Other institutes facing a similar cut of 10 per cent in personnel include the German air and space research centre (DLR), the biotechnology research centre (GBF), the environment and health research centre (GSF) and the institute for plasma physics (IPP). Each hopes to avoid forced redundancies, which follow 10 years of smaller cuts that have chipped away at their missions, and none has specific plans to change research priorities. "We are now having to eat into our own flesh", says Richard Radlof of the GBF, which is losing 30 full-time positions.

Thomas Köstlin, head of administration at the plasma physics institute, says that staff has been trimmed each year since 1980. Some positions were returned, however, and the exercise served to circulate scientists between research establishments. "But since we only have one topic", he says, "it is more difficult for us to receive people back." The institute must now lose 96 — about a tenth — of its present staff, and the cuts mean that the start of construction of a Wendelstein 7X accelerator will be delayed until at least 1995.

But the biggest blow falls on the four centres whose areas of research are deemed to be no longer interesting. By the end of this year, the society for exploration of nuclear energy in shipping will lose its underwater technology department, which was developed 15 years ago on the faulty assumption that German industry would require such expertise.

The GMD, the centre for mathematics and data handling near Bonn, is to lose 100 of its staff of 1,300. In addition, its budget has been cut drastically following the failure of an important co-operative project with industry relating to parallel computing, and the centre is shifting resources into applied research.

The two largest national research centres, KfK and KFA, each with more than

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Hahn-Meitner's heavy-ion accelerator will soon cease being a research tool.

4,000 staff, are under the greatest pressure to abandon their original mandate to foster nuclear technology. With budgets that have been squeezed hard for the past decade, they have been ordered to lose up to 20 per cent of their staff in the next three years and to find worthwhile projects that do not involve non-safety aspects of nuclear technology research. Concerned about what may happen if they do not demonstrate greater accountability, they nevertheless face the difficult job of retraining scientists whose *raison d'être* had been a single research project.

The new smaller national research centres hope that a new management approach giving them greater flexibility will help them to survive the current economic crisis. Such a system would replace the current system of evaluations, which the research ministry believes do not provide sufficient accountability. **Alison Abbott**

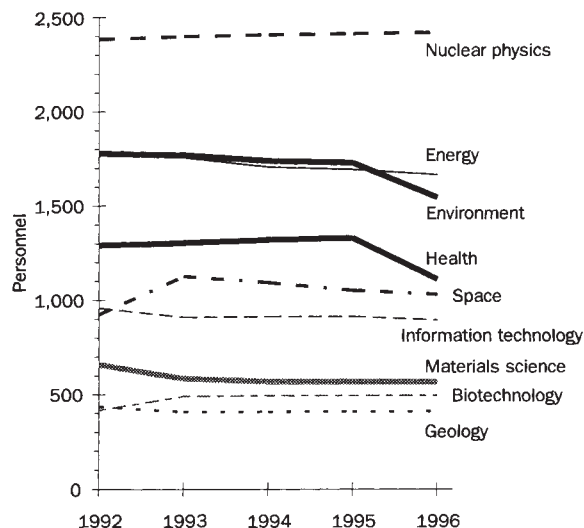
OTA tackles gene patents

Washington. A panel of legal experts met last week at the congressional Office of Technology Assessment (OTA) to mull over the legal implications of patenting human DNA sequences. Although the one-day meeting raised more questions than it answered, panel members felt that the challenge for such applications is meeting the legal standards of 'utility' and 'non-obviousness'.

One of the most troubling questions raised at the meeting was the effect of granting patent rights for partial human DNA sequences on patents for full-length genes whose biological function has been elucidated. It is feared that companies prevented from making such patent claims would be reluctant to invest in research in this area.

OTA will evaluate the commercial, ethical, legal and social implications of patenting human DNA sequences in the wake of a 1991 filing by the National Institutes of Health for patents on thousands of human DNA sequences of uncharacterized function. In July it will consider the effect of such actions on international research. Its report is due next spring. **Diane Gershon**

Staffing levels at national research centres



Source: German Ministry of Research

Support grows for UNESCO but money remains a problem

London & Washington. Supporters of the United Nations Educational, Scientific and Cultural Organization (UNESCO) see growing evidence that the US and British governments will rejoin an organization that they left nearly a decade ago after complaining of excessive politicization and chronic mismanagement. But they concede that the cost of membership may be too high a hurdle to clear.

Their optimism is based on statements by government officials that, even though major problems still exist within the organization, the end of the Cold War and recent management reforms have largely met the chief ideological and practical objections. In Washington, the US State Department is about to begin a two-month inter-agency review to gauge the potential benefits of rejoining after a nine-year absence. This follows a report last year from the General Accounting Office and a recent visit to UNESCO's Paris headquarters by a congressional delegation, both of which concluded that the organization has done much to correct previous shortcomings.

Doug Bennet, assistant secretary-designate for the State Department's Bureau of International Organization Affairs and the leading administration figure in reaching an eventual decision, is said to be sympathetic to UNESCO. At the same, its friends in Congress are mustering support for non-binding resolutions calling for the United States to rejoin before its biannual general conference in Paris in October.

The resolution, introduced last week by former UNESCO ambassador Esteban Torres (Democrat, California), has attracted the support of former opponents, including Tom Lantos (Democrat, California). "UNESCO has now cleaned up its act," says Lantos, chairman of a House of Representatives subcommittee on international security, international organizations and human rights. "But when we get back in, we will watch both its politics and its fiscal performance with great care."

In London, Baroness Chalker, the Min-

ister for Overseas Development, is coming under pressure to act from both Houses of Parliament. A resolution calling for Britain to return to an organization that it helped to create in 1946 has so far been signed by 214 (out of 650) Members of Parliament, including 22 Conservatives.

UNESCO's director-general, Spanish biochemist Federico Mayor, was given a relatively sympathetic hearing last week when he outlined some of the body's recent changes to the House of Commons Select Committee on Foreign Affairs. These changes, he said, include a further streamlining of operational programmes and more staff cuts at its Paris headquarters, which last year abolished 74 posts.

Mayor told the parliamentary committee that UNESCO's chief role "is in the transfer and sharing of knowledge" rather than to provide technical assistance or to sponsor research. He also said that plans have been scrapped for an "international communications order" — a controversial proposal that Western democracies saw as an attempt to censor the media.

However, the British government is not as yet convinced that the recent changes are as radical as UNESCO officials claim. Speaking in a parliamentary debate in March, for example, Chalker said there had been little progress in decentralizing activities away from Paris, that UNESCO's programmes were still insufficiently focused and that a decision to increase support for a "participation programme" — in which money is given to poorer member states to spend as they wish — was "quite mad". She also expressed concern that multilateral support in the developing nations is often less effective than bilateral programmes.

Nevertheless, Chalker has also said that her main reservation about applying to rejoin is financial. In the absence of new funding from the Treasury, the costs of membership — estimated at £10 million (US\$15 million) next year — would require cutting back other programmes financed by the Overseas Development Agency.

The United States may also find it difficult to justify an annual fee of \$55 million. Representative George Brown (Democrat, California), who is the chairman of the House Science, Space and Technology Committee, concedes that "developing trends in Congress against spending money and against international cooperation" will work against UNESCO membership. However Brown, who supports re-entry and has signed the Torres resolution, says that he remains "personally optimistic" that the Clinton administration will decide to rejoin before October.

David Dickson & Colin Macilwain

India drops useful forecast of monsoon

New Delhi. The Indian government will not this year issue a forecast of the severity of the summer monsoon, which has an enormous impact on the nation's economy, even though the model it has used for the past five years has been quite accurate. The government defends its decision on scientific grounds, but others see it as an attempt to play down the contribution of an influential scientist who developed the model and who earlier this year retired from government service.

The Indian Meteorological Department (IMD) says that the forecast has been halted because data for half of the 16 parameters used in the model are not available in time to be helpful. "Predictions in March or April based on incomplete data can at best be described as intelligent guesses but they are not scientific", says N. Sen Roy, IMD director general.

The model was developed in 1987 by Vasant Gowardiker, a chemical engineer who at the time was secretary of the Department of Science and Technology (DST), which controls the meteorological service. In 1991 he became science adviser to the prime minister, but in March he reached the mandatory retirement age of 60.

The Gowardiker model uses global and regional weather parameters such as the occurrence of El Niño to predict the amount of rain in the four-month monsoon season that begins in early June. Rain well below the normal 860 mm usually results in a poor harvest, and advance knowledge would greatly affect economic behaviour. In the past five years the forecast, issued in early spring to allow time for preparations either way, has proved to be quite close to the actual rainfall.

There is speculation that the model was abandoned because of persistent resentment in the weather department against Gowardiker, who is not a trained meteorologist and who was involved in building rockets for India's space programme before becoming head of DST. It may also be part of a wider problem in India (see *Nature* 362, 387; 1993) of professional jealousy and receiving credit for one's accomplishments.

Gowardiker points out that the government was willing for several years to use his model to make such 'guesses' and that it remains a useful tool. Without it, he says, "the country will be exposed to the greater danger of unfounded speculation and unscientific forecasts from all kinds of sources within and outside the country".

K.S. Jayaraman

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Dispute over money may foil US AIDS vaccines trial

Washington. An agreement between the US National Institutes of Health (NIH) and the Department of Defense (DoD) to test three therapeutic AIDS vaccines is about to collapse, with the \$20 million appropriated last autumn by Congress reverting to the DoD for other AIDS research work. NIH, which was to conduct the clinical trials under an agreement brokered last month by the White House (see *Nature* 362, 581; 1993), is ready to announce that it cannot afford to pay \$10 million to MicroGeneSys, the Connecticut company that supplies one of the three vaccines.

NIH scientists say that it would be "unprecedented" to pay a company for a product being tested for efficacy. Frank Volvovitz, president of MicroGeneSys, says his company will provide the vaccine at cost because "we're a small firm and we can't afford to do it otherwise".

The comparative trial proposed by NIH cannot go ahead without MicroGeneSys because Congress stipulated a "large-scale clinical investigation of the gp-160 vaccine" and the Connecticut company is the only US producer of gp-160. The other two proposed participants in the trial —

Genentech and Chiron, both of California — have promised to provide their gp-120 vaccines free.

The Army had originally planned to conduct a large-scale trial using only MicroGeneSys's product — called VaxSyn

"... we're a small firm and we can't afford to do it otherwise".

Frank Volvovitz
President, MicroGeneSys

— and a placebo. But complaints from AIDS activists and federal researchers led to an agreement that NIH would perform a comparative trial of three competing vaccines. The collapse of the agreement, and the accompanying public recriminations between Army and NIH researchers, will be seen as a failure of management on the part of the Clinton administration.

If NIH withdraws from the agreement, the \$20 million will revert to the Army's AIDS research budget, giving the Army

the right to proceed with a single-vaccine trial. But a more likely outcome is believed to be an extension of existing vaccine trials that the Army is planning in Thailand. The only vaccine to be used in the first stages of those trials is MicroGeneSys's gp-160.

An Army spokesman referred enquiries about problems with the agreement to NIH. "Our health affairs people have talked to NIH and the ball is in their court", the spokesman said.

NIH officials referred enquiries to the office of their political boss, health secretary Donna Shalala, where a spokesman said that the two departments were "still working out an agreement".

But people close to the negotiations said late last week that NIH might withdraw from the trial "any day now". They blame MicroGeneSys for insisting on payment and described the recent events as driving "the final nail in the coffin of scientific integrity of Army research".

VaxSyn is the oldest product to be offered as a possible therapeutic vaccine for carriers of HIV (human immunodeficiency virus), and it has been tested more extensively for safety and efficacy than any rival. But its value as a vaccine is a source of fierce scientific controversy, which increased after Congress appropriated money specifically for efficacy trials of gp-160 as a therapeutic vaccine.

Colin Macilwain

India moves ahead cautiously on US AIDS project

New Delhi & Washington. India's leading medical research administrator is trying to allay concern about collaboration with US scientists on preventive AIDS vaccine trials by promising that the trials will not take place without the permission of the Indian government.

Fears have been expressed at Pune in Maharashtra state, where the joint project is now identifying for future trials some 2,500 people at high risk of contracting HIV, that the sample may be used for tests of vaccines and other treatments that are not allowed in the United States. Such accusations have been denied by the US National Institutes of Health (NIH), which is financing the project.

Pune is one of nine sites in poor countries selected last autumn by NIH to receive \$3-million in awards in the first year of its Preparing for AIDS/HIV Vaccine Evaluation (PAVE) scheme. The programme matches US researchers with local teams to prepare samples of the population for trials to begin as early as mid-1994.

Most of the sites are in African countries with histories of similar work and little

chance of opposition. India, however, has until recently been sceptical of collaboration with US scientists.

The Pune project is a collaboration between the newly created National AIDS Research Institute (NARI) of the Indian Council of Medical Research (ICMR) and researcher Robert Bollinger of Johns Hopkins University in Baltimore, Maryland. Researchers hope to find 2,500 prostitutes and patients at clinics for those with sexually transmitted diseases and to develop a way to track them through future trials of vaccines and other treatments.

The Indian government has reaffirmed its policy not to allow any vaccine to be tested in India unless it has first been tested in its country of origin. ICMR director-general S.P. Tripathy says that the decision to prepare the sample does not imply permission for a trial. "The Indian government has to make its own decision whether or not it would allow the trial", he says. "There is a possibility that India may not undertake a trial at all."

US officials expect to face problems with trials in India but insist that, according to

Bob Fischer, an international health officer dealing with PAVE at NIH, "nothing we do overseas won't be done in the US. And where people don't want trials, we won't have them."

Bollinger says that PAVE has been misunderstood in India. It is a series of preparations, not a clinical trial, he says, adding that Indian agencies maintain control of it despite \$800,000 in US funding.

Some 310 people are known to have died of AIDS in India since the first case of HIV was reported in 1986, and it is estimated that 500,000 Indians carry the virus. Half of the estimated 50,000 injected drug users in north-east India are believed to be infected and Korshed Pavri, a retired ICMR virologist, says that someone is infected every five minutes in Bombay's red-light district.

Last year, India created the National AIDS Control Organization to tackle the problem. But its \$100-million budget over five years — mostly obtained as a loan from the World Bank — will be spent on hospital equipment, advertising campaigns and condom promotion, not research.

K.S. Jayaraman & Colin Macilwain

Britain to spend £100 million on innovative manufacturing

London. Britain's Science and Engineering Research Council (SERC) this week announced a £100-million (US\$150 million) programme on innovative manufacturing to harness the academic community to the needs of manufacturing — a goal set out by the government in this week's White Paper (policy document) on the organization of science (see page 289).

The new programme, known as the Innovative Manufacturing Initiative, aims to focus strategic research in universities and other institutions on themes ranging from pharmaceuticals research to new manufacturing techniques in the construction industry. Included in SERC's plans are proposals to fund a hundred new academic positions. It also intends to create a number of new university-based interdisciplinary research centres.

Under the imminent splitting up of the SERC, responsibility for the initiative would be taken over by the new Engineering and Physics Sciences Research Council. A major role will also be played by the Economic and Social Science Research Council, which is particularly keen to encourage the application of its research into the processes of industrial innovation.

However, in a strategically significant move, the detailed definition of the individual research programmes will be left to industrial participants. The steering committee, which is preparing proposals to be submitted to the full council in October, is chaired by Stuart Miller, director of research and technology at Rolls-Royce.

The new initiative follows a report on

engineering science prepared for the SERC by a special committee headed by Bob Malpas, chairman of the Cookson Group. It identified two strategic priorities for engineering research programmes funded by the council: generating a 'coincidence of purpose' between industry and the academic community and 'managing the interface' between the two.

"We want to steer the scientific community towards strategic research which is both industrially relevant and academically challenging," says Miller. "Our aim is to provide a structure for exercising an 'industry pull' on the SERC, and, through the council, on the academic community."

The SERC intends eventually to contribute about £30 million a year to the new initiative by refocusing the budget of its engineering directorate on research themes proposed by the steering committee. Some of the money is being found through cut-backs in other areas of research, such as semiconductor devices. SERC officials are optimistic that another £20 million will come from other government departments, in particular the Department of Trade and Industry, through schemes such as the LINK programme. And they hope that industry will match the £50 million expected from the government.

The Confederation of British Industry praised the initiative as "just the type of partnership" that industry is seeking with the research community. University scientists are more apprehensive, fearful that there will be less scope for research with no short-term applications. **David Dickson**

Europe is split over combining defence research

Paris. A proposal to merge European defence research into a single agency finds France and Germany on one side against Britain.

The creation of a European Armaments Agency (EAA) would go some way to forging a common defence research policy and a single European defence industry, say French and German officials. Bureaucracy could be avoided, they say, by giving the EAA its own budget and modelling it on the US Advanced Research Projects Agency (ARPA).

But Britain is not willing to go that far. Speaking earlier this month at a two-day conference on "Science and Defence", Geoffrey Pope, deputy chief scientific adviser at the British Ministry of defence, said that he welcomed looking at EAA as a possible means of managing existing multilateral agreements. Extending its role, he warned, would risk compromising national priorities, abandoning Britain's thrifty coupling of research to "real military needs" to the more profligate industrial policies of its continental partners, and weakening existing agreements with France, Germany and the United States.

Pope cites the EUCLID (European cooperation for the long term in defence) programme — set up by 13 European countries in 1989 to foster multinational cooperation in technology — as an example of how not to collaborate. Disagreement about participation and the financial contributions of each government have slowed progress to the point where the technologies being developed could be obsolete before the first projects are even completed (see *Nature* 357, 181; 1992).

Britain's chief concern is that the EAA is being created because of a desire to further the common defence and foreign policies envisaged in the Maastricht agreement rather than because it would improve existing arrangements. Europe's defence ministers are looking for ways to spread the increasing costs of defence research and strengthen the European arms industry. However, Pope reckons this would best be done not through a supranational agency but by increasing collaborations between Britain, France and Germany, which together account for more than four-fifths of Europe's spending on defence research.

France and Germany disagree. "It's not enough to work together only on top priorities", says Norbert Roy, an official in the German Ministry of Defence. "We need a balanced joint R&D programme... we need to develop a supranational policy. Germany is willing to give up its autonomy in defence research", he says. **Declan Butler**

African women form group to increase links

Washington. A new association hopes to improve communications among the small number of African women scientists and increase opportunities for African girls to enter science. The Association of African Women in Science will be chaired by Deborah Enilo Ajakaiye, professor of physics at the University of Jos in Nigeria, who says that it will "work to reach a critical mass of women in science in Africa".

Ajakaiye says that such an organization would eliminate the current situation in which she must, for example, communicate with colleagues in Botswana via the Commonwealth Institute in London. "We need something of our own", she says. The idea was discussed last week at a symposium organized by the American Association for the Advancement of Science.

The initial goals of the association, Ajakaiye says, will be to gather data on the

numbers of African women at various levels of science, to document indigenous technologies used by African women and to encourage the production of basic educational material to popularize science among schoolgirls. The group is seeking financial support to carry out these goals as well as to hold additional meetings at which members can share their experiences.

Lydia Makhubu of the University of Swaziland, president of the Third World Organization for Women in Science, told the forum that the under-representation of women "deprives the continent of a substantial input and amounts to a neglect of 50 per cent of the human potential". African women in the United States helped to form the association, and its first secretary is Josephine Beoku-Betts, an assistant professor of sociology at the University of Georgia, Athens. **Colin Macilwain**

Drawbacks of peer review

SIR — All reputable learned journals employ the peer-review system to help to decide on publication or rejection of submitted articles. Thus the system can be viewed as a method for evaluating the quality of potential publications. Every scientific method should be validated before it is generally used. One essential step in this validation is the assessment of the method's reproducibility. We have attempted to define the reproducibility of the peer-review method.

Copies of one paper submitted to a medical journal were sent simultaneously

the whole range. The paper sent out for review was by no means on a controversial subject. The results therefore suggest that the method of evaluating scientific papers by peer review is unreliable and open to bias and should itself be submitted to evaluation.

Since acceptance or rejection of a paper nowadays strongly influences the career of the authors, the poor reproducibility is a cause for concern. One would therefore hope for an improvement of the method. A system incorporating regular, blind tests concerning the quality of the review-

graph were obtained on 8 April and subsequent observations with the Faint Object Spectrograph were secured on 15 April.

The high resolution observations species superimposed on the spectrum of the supernova. The data are fabulous and reveal a wealth of information on the distribution of gas in our Galaxy and in M81. The lower resolution observations, which I have not seen, were taken to study spectral features in the supernova itself. I believe that they were successful.

The article in News and Views concludes with a discussion of nearby type I supernovae and gives the mistaken impression that no type I supernova has been near enough to give an unequivocal calibration of the distance scale.

Last year, Sandage and collaborators (*Astrophys. J.* **401**, L7; 1992), using the Hubble Space Telescope, discovered and measured 27 Cepheid variables in the galaxy IC4182, thereby establishing a calibration for the type Ia supernova SN1937C. They report a Hubble constant of $51(\pm 10)$ km s⁻¹ Mpc⁻¹.

Chris Blades

*Space Telescope Science Institute,
3700 San Martin Drive,
Baltimore,
Maryland 21218, USA*

□The important study by Sandage *et al.*, valuable though it is, has not definitely resolved the question of Hubble's Constant. — Editor, *Nature*.

Descriptive statistics for each quality criterion

	Frequency of ratings					q1	q3
	U (1)	A (2)	F (3)	G (4)	E (5)		
Scientific merit	2	3	7	14	5	3.00	4.00
Clarity of description	1	0	6	15	8	3.75	5.00
Statistical methods	4	2	7	11	7	3.00	4.00
Methodology	2	0	5	17	6	3.75	4.00
References	0	2	2	15	11	4.00	5.00
Quality of tables/ figures	0	1	10	16	3	3.00	4.00
Discussion	1	3	8	13	5	3.00	4.00
Linguistic merit	0	1	6	16	7	3.75	4.25
Overall judgement	2	2	4	16	5	3.00	4.00

U, unacceptable (1); A, acceptable (2); F, fair (3); G, good (4); E, excellent (5).

q1 = 25% percentile (first quartile), q3 = 75% percentile (third quartile) median and mode for all criteria: 4 (frequencies of median and mode are printed in bold)

to 45 experts, all of them members of editorial boards of journals relating to the subject of the submitted paper. They were asked to express their opinion of the paper on the journal's standard questionnaire judging 8 quality criteria on a numerical scale from 5 (excellent) to 1 (unacceptable). In addition, an overall judgement of the paper had to be indicated on the same scale. No reviewer was told that he or she was being tested. Thirty-one adequately filled forms were received back; 14 potential reviewers declined because they claimed to be not fully competent or had too little time to do the work within the deadline. On the basis of the individual ratings, the reproducibility was estimated by calculating descriptive statistics for each item. The results are depicted in Table 1.

They demonstrate disappointingly poor reproducibility with extreme judgements ranging from 'unacceptable' to 'excellent' for most criteria. Even the criterion 'linguistic merit', which could have been expected to yield a fairly uniform judgement because the paper was written by two native English-speakers from the United Kingdom, was no exception. Similarly the overall judgement rating was spread over

ers might exclude some sources of publication bias (1–3). The absence of reliability suggested by the present findings seems unacceptable for anyone aspiring to publish in peer-reviewed journals.

E. Ernst

T. Saradeth

K. L. Resch

*Department of Physical Medicine
and Rehabilitation,
University of Vienna,
Währinger Gürtel 18-20,
1090 Vienna, Austria*

1. Ernst, E. & Kienbacher, T. *Nature* **352**, 560 (1991).

2. Wilmhurst, P. *Lancet* **337**, 1419 (1991).

3. Ernst, E., Resch, K. L. & UUher, E. M. *Ann. int. Med.* **116**, 958 (1992).

HST still alive

SIR — I am writing to correct the impression (*Nature* **362**, 585; 1993) that the Hubble Space Telescope (HST) has not observed the recent bright supernova SN1993J in M81. Two separate observing programmes were initiated with HST within days of discovery of the supernova. The first observations with Hubble and the Goddard High Resolution Spectro-

Aristotle's origins

SIR — On the cover of the 7 January 1993 issue of *Nature* you refer to the Greek philosopher Aristotle as "Athenian philosopher and logician. . .". Aristotle was not from Athens. According to the *Encyclopedia Britannica*, he was born (384 BC) and raised in the Greek colony of Stagira in Macedonia, northern Greece. Although he spent several years in Athens studying and teaching, he always remained a Macedonian. Indeed, he was forced to leave Athens shortly after the death of his student Alexander the Great (323 BC), because of rising anti-Macedonian sentiments in Athens². Please notice that Macedonia, the largest province of Greece, is not to be confused with the pseudo-"Macedonian" state that emerged in 1991 after the break-up of Yugoslavia.

George Philippidis

*90 Corona Street,
Apt 1003,
Denver,
Colorado 80218, USA*

Letters submitted for Correspondence should be typed, double-spaced, on one side of the paper only.

Bringing photosynthesis to the bench

A series of experiments with genetically engineered chromatophores from purple bacteria suggests an unexpected coupling between molecular vibrations and the electron transfer at the heart of photosynthesis.

Most of the understanding of the electronic properties of molecules accumulated in the past six decades rests on what is known as the Born–Oppenheimer approximation, the idea that the motions of atomic nuclei are so much slower than the characteristic times of electronic motions that the nuclei can be regarded as fixed in position while one calculates the electronic states. Quite apart from its plausibility in many circumstances, the assumption is also a great convenience: it allows the solution of problems that would otherwise be intractable. Quantum chemistry has flourished marvellously as a consequence.

But, as always, there must be exceptions. In the broadest sense, the problem tackled by Jean-Louis Martin and his colleagues at the INSERM Applied Optics Laboratory and described on page 320 of this issue is one of these. They are concerned with the process of charge transfer following the absorption of light quanta by the reaction centres of the photosynthesizing purple bacteria. The light-collecting elements consist of four molecules of bacteriochlorophyll, linked, together with quinone molecules, to electron acceptors known as bacteriopheophytins. There are specific protein appendages as well.

The reaction centres or chromatophores are embedded in membranes and, in the real-life photosynthetic process (as in that induced by the more familiar plant chlorophyll), the first step is the apparently irreversible transfer of electronic charge across the membrane. In bacteriorhodopsin, this process is known to take 3 picoseconds ($3 \cdot 10^{-12}$ seconds) at ordinary temperatures, perhaps only a quarter of that time at 10 K.

What Martin and his colleagues have shown is that this reaction time is comparable with and even less than that for which the motion of the atomic nuclei of the protein parts of the reaction centre are coherent with each other. In other words, it may be inferred, there are molecular vibrations whose timescale is comparable with the time taken for the electron transfer process, and which therefore cannot be left out of account in discussions of the first step in bacterial photosynthesis.

This is a technical *tour de force*, combining esoteric techniques from laser spectroscopy with those from genetic engineering. Thus the study has been made possible only by the genetic engineering of one of the protein components of the reaction complex so that actual electron transfer does not take place; instead, on the absorption of a light-

quantum, the complex remains in an excited state whose properties can be followed for several picoseconds.

The essential observation, that the fluorescence of this excited state seems to be periodic on a picosecond scale, was first published 18 months ago (*Proc. natn Acad. Sci. U.S.A.* **88**, 8885–8889; 1991). One interpretation of that result was that the periodicity of the fluorescence is indeed a consequence of the coherent molecular vibration of one of the protein components of the photosynthetic reaction complex. The new development is that the interpretation has been shown to be correct.

That may sound simple enough, but it is not. For one thing, to study phenomena lasting for a few picoseconds, it is necessary to excite them by means of laser pulses whose duration is even shorter, lasting for just femtoseconds (where a femtosecond is 10^{-15} seconds). Similarly, the analysis of the fluorescence of the excited complex must be comparably sensitive in time (which is where the novelty of this account of the research resides). But verisimilitude also requires that the bacterial reaction complex should be embedded in real bacterial membranes, while the need that the signal should not be buried in the noise requires measurement at 10 K. That could well have been a recipe for a small army of researchers, not just five of them.

What the authors do is to excite the bacterial reaction complexes with a femtosecond pulse of laser light centred on a near-infrared wavelength between 850 and 900 nm. Because the duration of the pulses is a few tens of femtoseconds, the effective spread of wavelength (which is inversely proportional to the duration of the pulses) is always great enough to span the absorption band. Oscillations in the output are apparent to the eye in the fluorescent output. Fourier transforms of the output then yield the frequencies of the underlying vibrations which are supposedly their origin.

It is striking that the spectroscopy is evidently good enough for the authors to engage in discussions of their data comparable in detail (and complexity) with those familiar in the spectroscopy of simple diatomic molecules. The vibration that seems to matter most is that whose wavenumber (a proxy for frequency) is 70 cm^{-1} . (There is another frequency at 15 cm^{-1} that also seems repeatedly to crop up.) That is well into the infrared, as the frequency of a molecular vibration should be.

The similarity between the time-span of the electron transfer process and the time-

span of the vibrations of the protein molecules may be fortuitous, but that is to beg the question that interests others as well as Martin and his colleagues. Is it just an accident that the molecular vibration has just the frequency to confound the fluorescent output of the excited state that would normally result in electron transfer? Or is it possible that the molecular vibration is an essential part of the process, perhaps contributing to the very high efficiency of the electron transfer?

For what it is worth, the authors seem (appropriately judiciously) to be leaning towards the idea that the vibrations do indeed impel the process of electron transfer. Certainly the vibrations, which identify themselves by the relative persistence, appear to be unusual in their freedom from the dissipation they might be expected to suffer from. But it is far from clear just what vibration mode of a protein molecule, which has no direct part in the gathering of light, could play this trick.

Evidently it is within the bounds of possibility that these questions could be adjudicated by experiment. If it has been possible by genetic engineering to produce reaction complexes that do not allow electron transfer, why not alter the constitution of the protein molecules involved in such a way that their vibrational spectrum is changed and then see whether the efficiency of the transfer is reduced? That task seems well within the competence of Martin and his colleagues.

But what proteins to alter, and in what way? The trouble, as things stand, is that there is no way of calculating the vibrational frequencies of such a complicated structure as the reaction complex, especially within its natural environment of a membrane structure. Molecular dynamics or some variant of it seems the best way forward, but only with some difficulty.

Of course, on the assumption that the protein vibrations are indeed some kind of driving force for the electron transfer, it may be possible to guess at the symmetry or some other property of the vibration by estimating the transition probabilities between the excited state and that with a transferred electron, but that would require an element of luck. In the circumstances, it is commendable that Martin and his colleagues do not claim a big step forward in the understanding of photosynthesis. But it is an important step forward in that it could become the springboard for a big step.

John Maddox

Seeing the tree for the woods

Alan Cowey

WE can all seek and pick out a face in a crowd, select a particular object from an array, attend to one voice or conversation at a noisy party. This extraordinary skill was ignored by many early psychologists whose roots were in British empiricist philosophy and its preoccupation with sense data, a point of view that left little room for inner control and selection in perception. As usual the inimitable William James put his finger on the problem¹. Writing in 1890 about visual attention he describes how it "may come about either by reason or by a stimulus from without, or in consequence of some unknown inner alteration; and the change it brings with it amounts to a concentration upon one single object with exclusion of aught besides . . .".

Here James introduced the twin ideas of selecting certain sense data and actively suppressing others. More than a century later, on page 345 of this issue², Chelazzi *et al.* describe an experiment that sheds light on the neural basis of selective attention and on the points first raised so clearly by James.

Of course, experimental psychologists were not idle in the intervening years and amply demonstrated the powerful effects of voluntary attention on what we perceive, for example our increased sensitivity to faint signals when we know the position where they will appear. Such experiments laid the ground for physiological investigations of selective attention but, until recent experiments of the kind reported by Chelazzi *et al.*, progress was meagre. Why?

By definition, voluntary attention requires an alert mind and it was only in the 1950s that physiologists began to record from within the brains of awake animals. It was at this time that a series of experiments³, which provoked enormous interest, showed that the gross brain potentials in the cat evoked by stimuli in one modality (for example responses in the cochlear nucleus to sounds) were attenuated when the cat attended to another modality (for example watching a mouse). Unfortunately this selective peripheral filtering and inhibition was a mirage, because the changes could often be attributed to postural adjustments by the animal and similar changes occurred even in the modality being attended to. Comparable studies of event-related potentials in human subjects reveal changes in latency and amplitude that correlate with voluntary attention, but do not uncover precisely what is going on in the brain.

One way of avoiding the problem is to record from individual brain cells in

behaving animals, and the enhancement effect reported first by Goldberg and Wurtz⁴ seemed an excellent model of selective visual attention. These investigators found that, when a light was presented in the receptive field of a cell in

V4, Moran and Desimone⁵ found that the response of a cell to its preferred stimulus was reduced if the monkey was paying attention to another visual stimulus that also lay within the receptive field. This was the first clear demonstration of events in a single cell in relation to selective attention to features of a stimulus other than its position.

From there it was but a short step to the latest experiment². Chelazzi *et al.* trained monkeys to fixate a spot and briefly superimposed a complex shape, which would then reappear somewhere else and had to be fixated. If the shape was one that excited the cell from which they were recording, the elevated discharge continued while the monkey was also waiting to move its eyes to the same target shape when it subsequently appeared along with other irrelevant shapes, all within the large receptive field of the cell. However, if the brief shape was one that did not excite that particular cell and it was now the next target, the previous shape, now present as one of the irrelevant distractors but still within the receptive field, no longer effectively excited the cell. In other words, the response of the cell to a shape in its receptive field depends on whether the monkey is looking for it.

The new report comes closer than any other to demonstrating some of the nuts and bolts of William James's "inner alteration" and its consequent "concentration upon one single object with exclusion of aught besides". And it comes with an unexpected bonus. It was James again who pointed out that "The only things which we commonly see are those that we preperceive and . . . which have been labelled for us and the labels stamped into our mind. If we lost our stock of labels we would be intellectually lost in the middle of our world". The cells described by Chelazzi *et al.* reside in the temporal lobe, where damage in monkeys leads to a condition akin to human visual object agnosia. Is this because what James called preperceptions and labels — both necessary for selective attention in visual search because we have to know what it is we are looking for — also require, and might be mirrored in, the activity of cells of the inferior temporal cortex? How these cells acquire their "attentive" properties is not yet clear, but that search is on too. □

Alan Cowey is in the Department of Experimental Psychology, University of Oxford, South Parks Road, Oxford OX1 3UD, UK.

Mental marvel — pick a face, and exclude aught besides.

the midbrain of a trained monkey, the response was enhanced when the monkey was required to move its eyes to the light. As the enhancement preceded the eye movement, it seemed reasonable to relate it to the animal's presumed attention to this part of space. Although this is correct in some instances, the enhancement effect is not always confined to that part of space nor is it always dissociable from preparing to move the eyes.

What was needed was a 'purer' test of attention, and it came from the laboratory of the present authors in 1985. While recording from cells in visual cortical area

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1. James, W. *The Principles of Psychology*, Vol. 1 (Henry Holt, New York, 1890).
2. Chelazzi, L., Miller, E. K., Duncan, J. & Desimone, R. *Nature* **363**, 345–347 (1993).
3. Hernandez-Peon, R., Scherrer, H. & Jouvett, M. *Science* **123**, 331–332 (1956).
4. Goldberg, M. E. & Wurtz, R. H. *J. Neurophysiol.* **35**, 560–574 (1972).
5. Moran, J. & Desimone, R. *Science* **229**, 782–784 (1985).

Too hot for earthquakes?

Douglas A. Wiens

THE largest earthquakes, for example that in Chile in 1960 and another in Alaska in 1964, occur at subduction zones, where the sea floor slides under the continents. But other nearly identical subduction zones seem to be relatively benign. Why the difference? Tichelaar and Ruff¹ and Hyndman and Wang², in a pair of new papers, suggest that the temperature structure along the thrust interface may be the significant factor in determining whether the two plates lock and intermittently slip, or whether they slide smoothly past one another.

What makes some subduction zones seismically active and others quiescent is of particular interest to those living near the Cascadia subduction zone, which extends along the coast from northern California to southern British Columbia. In historical times, there have been no large earthquakes in the region, but there is some geological evidence for possible quakes before the arrival of European settlers³. One option is that the overlying continental plate and underlying oceanic plate are locked rigid, and have been accumulating strain since the last earthquake, in which case the strain may be released by an earthquake as large as the Chilean earthquake of 1960⁴ (magnitude $M = 9.5$). Alternatively, the two plates may be moving effortlessly past one another. The difference, as far as those living nearby are concerned, couldn't be greater.

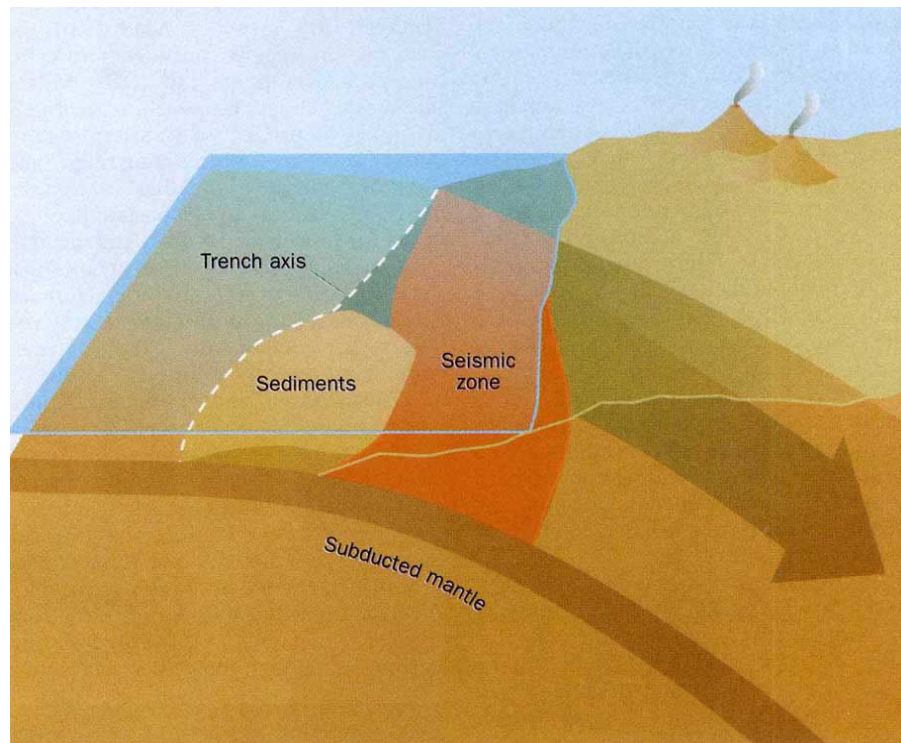
The two new papers start from a consideration of the width of the zone of seismic activity (see figure), which sets the maximum rupture size. The upper extent of the seismic zone seems to be limited by a region of stable sliding, where unconsolidated sediments on the downgoing plate are scraped off by the overlying plate. No earthquakes are initiated in this region, and despite evidence⁵ that slip occurs along the base of the sediment pile during large, shallow events in the subduction zone, little energy is released there.

Tichelaar and Ruff set out to determine whether the lower extent of the seismic zone is controlled by a critical temperature above which seismic rupture does not occur. This seems a reasonable assumption, as the maximum depths of earthquakes in the interiors of plates (where the temperature structure is more readily determined) are clearly limited by temperatures of about 350 °C in continental crust⁶ and 750 °C in the oceanic upper mantle⁷. These limiting temperatures are often ascribed to the onset of thermally activated 'power-law' creep, plastic behaviour which generally occurs at lower

temperatures for continental crustal rocks (silica rich) than for oceanic upper-mantle ones (iron–magnesium rich). An extension of this idea is that the behaviour of faults is controlled by frictional stability transitions^{8,9}, so that the change from stick-slip behaviour to stable sliding occurs at about 300–350 °C and the transition from stable sliding to ductile

the limiting depth can be inferred using a simple thermal model for subduction zones¹⁰, constrained by heat-flow observations at the surface. Tichelaar and Ruff suggest that the lower edge of the seismic zone does turn out to be controlled by temperature, but that the temperature varies from 400 to 550 °C for different subduction zones.

Hyndman and Wang apply their version of thermal control specifically to the Cascadia subduction zone, at which the subducted lithosphere is exceptionally young (having been formed just 8 million years



Cross-section through a subduction zone, showing the seismically active region along the shallow thrust zone between the plates. The upper extent of the seismic zone is limited by contact with the soft sedimentary pile, and the lower extent is presumably limited by high temperatures which inhibit faulting (after Tichelaar and Ruff¹).

behaviour occurs at about 450 °C in the continental crust. The first transition would correspond to the limiting temperature for rupture initiation, but once initiated, the rupture could propagate into material with temperatures below the second transition.

Tichelaar and Ruff estimate the limiting temperature for several subduction zones by first locating the depth along the thrust zone where seismic rupture ceased. Because it is difficult to determine the depth to which ruptures extend during very large earthquakes, they infer the limiting depth through analysis of seismic body waveforms from smaller earthquakes that have occurred near the downdip edge of the seismically active thrust zone. This procedure involves a significant assumption that the deepest smaller earthquakes also mark the depth limit for the rupture of the larger earthquakes. The temperature at

ago at a mid-ocean ridge a mere 250 kilometres away) and is covered with thick, insulating sediment. They assume that the rupture zones of large earthquakes would be limited by a 450 °C temperature, as indicated by rock-mechanics experiments on continental crustal rocks^{8,9}, and perform detailed thermal modelling of the Cascadia subduction zone to estimate the width of the seismic zone. They find that the top of the downgoing plate is at a temperature of 250 °C before it even enters the subduction zone. Off Vancouver Island, the calculations indicate, the seismic zone is about 100 km wide and located seaward of the coast. The seismic zone becomes somewhat wider and extends slightly onshore in the Olympic mountains region, owing to an older plate age and a shallower dip angle. Nevertheless, the width of the seismic zone throughout the Cascadia

zone is considerably less than in typical subduction zones. If the authors are right, the good news is that the narrow seismic zone limits the possible size of earthquakes in Cascadia and that the offshore location will reduce the damage to populated areas compared with most subduction zones, where the locked zone may extend onshore.

Should the residents of the Pacific Northwest rest easy? That would be premature, although these studies are important in providing physical models for the variability of subduction-zone seismicity. First of all, problems both in locating the lower edge of the rupture zone and in the thermal modelling leave the observational evidence for a cutoff temperature pretty uncertain. The main difficulty comes in estimating the frictional heating properties of the fault itself, which are poorly constrained owing to a sparsity of heat-flow observations and poor knowledge of radioactive heat production in the crust. Tichelaar and Ruff have an alternative set of thermal models which are consistent with a cutoff of seismicity at 250 °C. On the other hand, another analysis¹¹ of the cutoff came up with a limiting temperature of 850 °C. And the limit of 450 °C assumed by Hyndman and Wang may not apply at depths of 30–50 km in subduction zones, where the downgoing slab will be in contact with rocks of the subcontinental mantle, which may permit faulting at higher temperatures.

Caution is also warranted because even a 100-km wide seismic zone off the coast of western North America could still produce a magnitude-8 earthquake, which would produce destructive shaking and damaging local tsunamis. It is also worth noting that the 1960 Chilean earthquake, the largest earthquake ever recorded by a seismograph, occurred in a region similar to Cascadia where the subduction zone should be hot and the seismic zone thin⁴. It would be reassuring if the thermal models could show us the difference between the two zones. □

Douglas A. Wiens is in the Department of Earth and Planetary Sciences, Washington University, St Louis, Missouri 63130, USA.

1. Tichelaar, B. W. & Ruff, L. J. *J. geophys. Res.* **98**, 2017–2037 (1993).
2. Hyndman, R. D. & Wang, K. J. *J. geophys. Res.* **98**, 2039–2060 (1993).
3. Atwater, B. F. *Science* **236**, 942–944 (1987).
4. Heaton, T. H. & Hartzell, S. H. *Science* **236**, 162–168 (1987).
5. Pelayo, A. M. & Wiens, D. A. *J. geophys. Res.* **97**, 15321–15337 (1992).
6. Chen, W. P. & Molnar, P. *J. geophys. Res.* **88**, 4183–4215 (1983).
7. Wiens, D. A. & Stein, S. *J. geophys. Res.* **88**, 6455–6468 (1983).
8. Tse, S. T. & Rice, J. R. *J. geophys. Res.* **91**, 9452–9472 (1986).
9. Scholz, C. H. *The Mechanics of Earthquakes and Faulting* (Cambridge University Press, 1990).
10. Molnar, P. & England, P. J. *J. geophys. Res.* **95**, 4833–4856 (1990).
11. Kao, H. & Chen, W.-P. *J. geophys. Res.* **96**, 21443–21485 (1991).

Rough stuff at Saturn

R. E. Johnson

THE first direct observation of the hydroxyl radical in the vicinity of the inner satellites of Saturn by Shemansky *et al.*, reported on page 329 of this issue¹, confirms previous suggestions that water molecules are being ejected from these icy bodies, but in amounts much larger than expected. The authors conclude that the stream of micrometeorites known to bombard the surfaces of Enceladus and Tethys², the satellites nearest to the observation region, must therefore be much larger than expected. Micrometeorite bombardment is already assumed to produce a transient vapour and plasma over Saturn's main rings³, and limits the lifetime of the rings⁴, so that the new observations have important implications for objects in Saturn's system. It is also intriguing that the report comes fast on the heels of the first detection of streams of dust from Jupiter's moons and interstellar space by the Ulysses spacecraft, recently reported in *Nature*^{5,6}.

The surfaces of Saturn's inner satellites and ring particles are known to be covered with water ice at temperatures of the order of 100 K. Because of this, the local plasma ions trapped in Saturn's magnetosphere⁷, as detected by the Pioneer and Voyager spacecraft, are thought to be formed from ionized fragments of water molecules ejected from these satellites, or from ionized fragments of nitrogen- and carbon-containing molecules that have

diffused inwards from the much larger satellite, Titan. The ambiguity in the plasma composition and, hence, in its source, came about because of the poor mass resolution of the Voyager plasma instruments. The Cassini mission, which is in preparation, will eventually tour this dynamic region carrying a plasma instrument with sufficient resolution to determine the masses and molecular character of the local plasma ions.

Although the observations of Shemansky *et al.* should lay to rest the Titan version of the model, the large amount of OH implied by their observations is a challenge to space physicists. The authors highlight the role that micrometeorites could play in eroding the surfaces of Saturn's icy satellites, although material ablated from the planet's main rings by micrometeorites may also contribute⁸. Other possible sources are sputtering of the satellites⁹ and E-ring¹⁰ grains by ions in Saturn's magnetosphere (Fig. 1), and even volcanism on Saturn's moon Enceladus (see Fig. 2).

If Shemansky *et al.* are right, the flux of material ejected from the icy satellites by micrometeorites must be much greater than previously realized. Micrometeorites, also called cosmic dust, are the small-scale debris from asteroids and comets, and can also originate from planetary satellites and interstellar space as shown by the recent Ulysses



FIG. 1 Saturn and its three main rings, the A-, B- and C-rings, as captured by the Hubble Space Telescope. The E-ring, invisible to direct observation, the minor moon Enceladus and the magnetosphere studied by Shemansky *et al.* are all about twice as far out as the main rings. Micrometeorite erosion and sputtering by ions in the magnetospheric plasma are important factors in the evolution of the satellite surfaces, ring particles and magnetosphere plasmas. Laboratory experiments in progress at the University of Virginia and elsewhere, mimicking the sputtering process, should help pin down source rates.

RÉSUMÉ

Sized up

Not only do female moths of the species *Utetheisa ornatrix* defer a decision on the paternity of their brood until after mating with a selection of suitors, but they choose between the sperm of each with a discrimination that surpasses understanding (C. W. LaMunyon and T. Eisner, *Proc. natn. Acad. Sci. U.S.A.* **90**, 4689–4692; 1993). The single significant determinant of this post-copulatory sexual selection is size: the bigger the male, the more likely it is that the female will choose his sperm to the exclusion of that of others. But why? It turns out that body size correlates with the size of the spermatophore and the strength of the males' alkaloid-based pheromone derived from the seeds of the moths' favourite food plant. This may indicate a larval preference for nutritious seeds rather than leaves — a favourable trait that could be inherited by future generations of moth.

Big difference

MEASURING the mass of the tiny neutrino is best done by observing the β -decay spectrum of tritium (^3H), which is converted into ^3He , an electron and Fermi's ghostly neutral particle. The mass difference of the nuclei is converted into the kinetic energy of the products, but needs to be known with extreme accuracy. R. S. van Dyck *et al.* have made the best measurement yet, trapping single ^3H and ^3He ions in a combination of electric and magnetic fields and timing their cyclical motion (*Phys. Rev. Lett.* **70**, 2888–2891; 1993). The mass of each is measured to within a billionth of an atomic mass unit. And the difference, 18,590 electronvolts, is accurate to just 1.7 electronvolts, which puts it in the ball park of most neutrino-mass measurements.

Wise wounds

ASSAULTS on epithelia are combated by repair processes of extraordinary efficacy and rapidity. If a wound is large, lamellipodia are thrown out from its edges; if small, its circumference narrows like an iris. Last year Martin and Lewis, writing in *Nature*, showed that the edges appear to be drawn in, as by a purse string. Now W. M. Bennett *et al.* (*J. Cell Biol.* **121**, 565–578; 1993) have brought a battery of microscopic and immunochemical methods to bear on this process, using an epithelial cell line as a model. Within 5 minutes, the borders of the wound (the purse string) begin to sequester an abundance of contractile elements — actin filaments, myosin II and tropomyosin, as well as villin and a tight-junction protein, ZO-1. The purse string evidently closes the wound and acts as a moving barrier, so as to preserve the distinction between the apical and basolateral cell surfaces. The cells can thus resume normal service with a minimum of interruption.

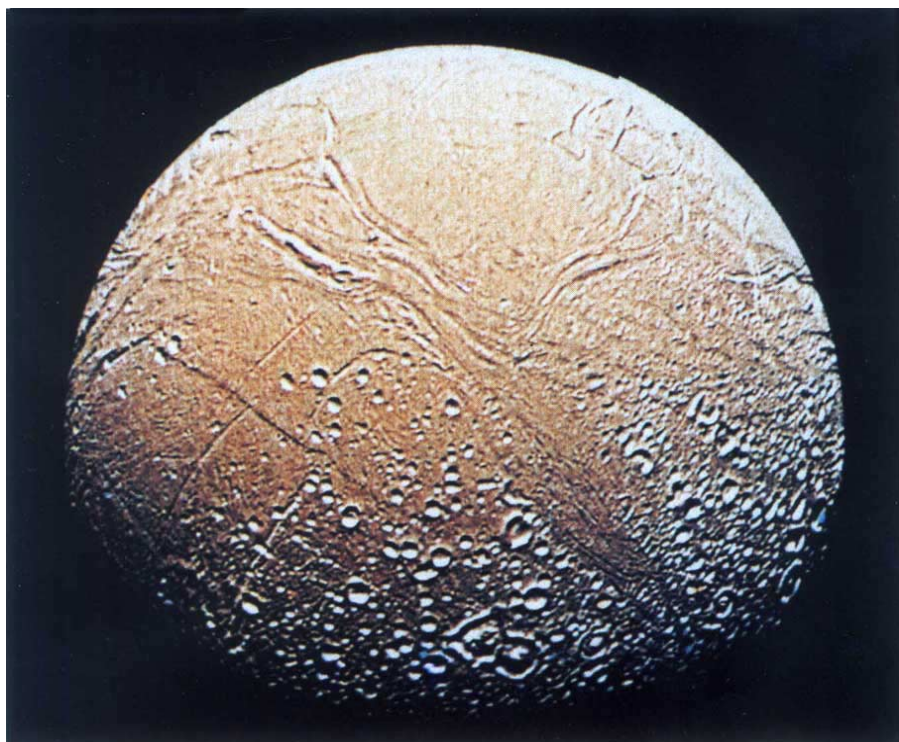


FIG 2. Enceladus's icy surface appears to be fresh and bright, which could be indicative of volcanic activity, although none has been observed directly. This volcanism has been implicated in the development of Saturn's tenuous E-ring, which, in turn, may be a source of ions and neutral species such as OH in the magnetosphere. Jupiter's moon Io is known to be actively volcanic, and is also thought to be a local source of dust and plasma.

observations^{5,6}. Their role in modifying the surfaces of Solar System bodies and their atmospheres is a source of great interest. Accelerated by gravity into a system like Saturn's or Jupiter's, they can collide at high speed with satellites and ring particles, ejecting fragments and vaporizing surface materials. Even grains from the lunar surface, collected during the Apollo missions, exhibit melting due to dust impacts. The flux of micrometeorites is also important as these particles erode Saturn's main rings, thereby supplying water to the planet's atmosphere and limiting the lifetime of the rings.

The apparently low value of the earlier estimates² of the micrometeorite-induced ejection rate can be attributed not only to uncertainties in the micrometeorite flux, but also to uncertainties in the amount of vapour ablated by each impact, and in the distribution in ejecta velocities. The last is particularly important, as it determines what fraction of the vaporized material escapes its source body's gravity and how far it is spread out. For instance, a fraction of the material eroded from the main rings adds to the neutral gas well beyond the edge of these rings⁸. The spatial extent depends on the tail of the velocity distribution of the ejecta, which is not known. So the physics of the impacts is at least as interesting as the mass flux of micrometeorites.

Curiously, there is very recent, Earth-

bound interest in particulate impacts as a means for producing, in a controlled way, gas-phase, intact biomolecules for laboratory analysis. The ejection of intact molecules by incident heavy-ions or by laser pulses is already a useful tool in biomolecular studies¹¹. But now huge molecular clusters, approaching the lower end of the micrometeorite size distribution, are being tried as impactors in some laboratories, because they are thought to be efficient at vaporizing a volume of material with relatively low internal energy. That the same physics of impacts should be important all the way from the harsh environment of the giant planets to the subtle conditions in biomolecular laboratories is indeed exciting. □

R. E. Johnson is in the Department of Materials Science and Engineering, University of Virginia, Charlottesville, Virginia 22903, USA.

- Shemansky, D. E., Matheson, P., Hall, D. T., Hu, H.-Y. & Tripp, T. M. *Nature* **363**, 329–331 (1993).
- Haff, P. K. & Eviatar, A. *Icarus* **66**, 258–269 (1986).
- Morfill, G. E. *et al.* *Icarus* **55**, 439–447 (1983).
- Northrup, T. G. & Connerney, J. E. P. *Icarus* **70**, 124 (1987).
- Grün, E. *et al.* *Nature* **362**, 428–430 (1993).
- Horanyi, M., Morfill, G. & Grün, E. *Nature* **363**, 144–146 (1993).
- Richardson, J. D. & Sittler, E. C. Jr. *J. geophys. Res.* **95**, 12019–12031 (1990).
- Pospieszalska, M. K. & Johnson, R. E. *Icarus* **93**, 45–52 (1991).
- Johnson, R. E. *et al.* *Icarus* **77**, 311–329 (1989).
- Morfill, G. E., Havnes, O. & Goertz, C. K. *J. geophys. Res.* (in the press).
- Johnson, R. E. & Sundqvist, B. U. R. *Phys. Today* March, 28–36 (1992).

Sunburnt fission yeast

Andrew W. Murray

ON page 368 of this issue¹, Walworth and colleagues offer insights into the feedback control that prevents cells with damaged DNA from entering mitosis. They have identified a gene (termed *chk1*, for checkpoint) which interacts both with *cdc2*, a gene that encodes the protein whose activation induces mitosis, and with genes whose products are involved in the detection of DNA damage. Their observations provide strong support for the idea that damaged DNA prevents entry into mitosis by controlling the activity of Cdc2 protein.

Passage through the G2/M checkpoint of the cell cycle is induced by the activation of MPF (maturation promoting factor), a protein kinase that is composed of Cdc2 and cyclin B. The feedback control that regulates passage through this checkpoint must consist of three sets of components: those that detect damaged or unreplicated DNA, a signalling pathway activated by these lesions, and enzymes that the signalling pathway regulates to control the activation of MPF (reviewed in refs 2 and 3).

Genetic analysis of the fission yeast has revealed two classes of mutants that allow cells with damaged or unreplicated DNA to enter mitosis. Members of the first class have no effect on the size at which undamaged cells activate MPF and enter mitosis, but prevent either unreplicated or damaged DNA from arresting cells in G2. These mutations — which include *rad1*⁻, *rad3*⁻, *rad9*⁻, *rad17*⁻ (sensitive to radiation) and *hus1*⁻ (sensitive to hydroxyurea, an inhibitor of DNA synthesis) — presumably identify mutants involved in the detection of DNA lesions and the transmission of this information to the cell-cycle engine⁴⁻⁶.

The second class of mutations disrupt the normal regulation of MPF activation and have two effects: they reduce the minimal size that cells must reach before they can enter mitosis and they abolish the ability of unreplicated DNA to prevent entry into mitosis. Most of these mutations affect the activity of the protein kinases and phosphatases that modify tyrosine 15 of Cdc2, a residue whose phosphorylation inhibits MPF activity. For example, overexpression of Cdc25, the tyrosine phosphatase, reduces cell size and allows cells with unreplicated DNA (but not those with damaged DNA) to enter mitosis^{7,8}. Inactivation of the two partially redundant protein kinases, Wee1 and Mik1, that are responsible for phosphorylating this residue, induces mitotic catastrophe: even when DNA replication is not inhibited cells enter mitosis at such a small size that they die, and inhibition of DNA replication does not block entry into

mitosis⁸. The molecular details of the mechanism by which unreplicated DNA modulates the tyrosine phosphorylation of Cdc2 are unknown. Although an earlier report suggested that Wee1-dependent phosphorylation of Cdc2 was required for radiation-induced G2 arrest⁹, more recent work demonstrates that $\Delta wee1$ strains can still arrest in response to DNA damage (A. M. Carr, personal communication).

The lethal consequences of simultaneously inactivating Wee1 and Mik1 can be overcome by mutations in *cdc2*, and it was during the investigation of one of these mutations that Walworth *et al.*¹ discovered a gene involved in the response to damaged DNA. They isolated high-copy suppressors of the cold-sensitive growth of a *cdc2*^{cs} mutant and showed that one of these, *chk1*, encodes a putative protein kinase. Deletion of *chk1* produced cells that grew normally but were sensitive to ultraviolet irradiation and failed to delay entry into mitosis when they were irradiated in G2. The mutant cells also fail to protect themselves against DNA damage caused by a temperature-sensitive mutation in DNA ligase. In contrast, the deletion mutants are not sensitive to hydroxyurea, suggesting that *chk1* is not involved in the response to unreplicated DNA. As Walworth *et al.* point out, it is hard to understand why a gene whose overexpression appears to stimulate MPF activity should also be a component of the machinery required to inhibit the entry into mitosis in response to damaged DNA.

Mutations in Cdc2 and the genes that regulate its activity seem to affect the response to either unreplicated DNA or damaged DNA but not both. Thus the dominant *cdc2-3w* mutation allows cells with unreplicated DNA to enter mitosis⁶ but has no effect on the response to DNA damage⁷, whereas $\Delta chk1$ cells have exactly the opposite phenotype¹. The phenotype of the *cdc2-3w* and $\Delta chk1$ mutations is considerably less severe than that of the *rad*⁻ and *hus*⁻ mutations that inactivate the mitotic feedback control: *rad1*⁻ cells are more sensitive to irradiation than $\Delta chk1$ cells and more sensitive to hydroxyurea than *cdc2-3w* cells^{1,4,5}. Mutations in *hus1* cause cells to die well before they enter mitosis⁶, implying that this gene is required to maintain the viability of interphase cells whose DNA replication has been inhibited, as well as preventing such cells from entering mitosis.

In mammalian cells DNA damage blocks the firing of replication origins until the lesions have been repaired¹⁰, suggesting that the Rad and Hus gene products may regulate the initiation of DNA rep-

lication in fission yeast. In addition, the feedback controls in fission yeast may regulate more than one step in the induction of mitosis. In budding yeast, mutations that prevent the tyrosine phosphorylation of Cdc28 (the homologue of Cdc2) do not abolish the response to unreplicated DNA, suggesting that some other steps in the induction of mitosis must be regulated by feedback controls^{11,12}.

Mutations that affect the response to damaged DNA have now been identified in both budding^{13,14} and fission yeast. None of the genes in one yeast has yet been shown to be homologous to those in the other, and the effect of preventing tyrosine phosphorylation of Cdc2 is different from that of preventing tyrosine phosphorylation of Cdc28. Although these discrepancies might mean that the feedback control that responds to damaged DNA is fundamentally different in these two organisms, I favour the possibility that, like most other parts of the cell cycle, the response to DNA damage has been conserved throughout evolution, albeit with some variation between organisms with different life styles.

This issue may be of more than academic interest. Mutations in the mammalian *p53* gene, which play a central role in human cancer, inactivate a feedback control that prevents cells with damaged DNA from entering S phase¹⁵. In patients with the inherited recessive disorder, ataxia telangiectasia, levels of *p53* do not increase in response to DNA damage¹⁶, implying that these patients' cells cannot detect damaged DNA. Perhaps some of the budding and fission yeast genes, whose products prevent cells with damaged and unreplicated DNA from entering mitosis, will turn out to have homologues in mammalian cells whose inactivation is involved in the genetic stability seen in human tumours. □

Andrew W. Murray is in the Departments of Physiology, and Biochemistry and Biophysics, University of California at San Francisco, San Francisco, California 94143, USA.

- Walworth, N., Davey, S. & Beach, D. *Nature* **363**, 368–371 (1993).
- Hartwell, L. H. & Weinert, T. A. *Science* **246**, 629–634 (1989).
- Murray, A. W. *Nature* **359**, 599–604 (1992).
- Al-Khodairy, F. & Carr, A. M. *EMBO J.* **11**, 1343–1350 (1992).
- Rowley, R., Subramani, S. & Young, P. G. *EMBO J.* **11**, 1335–1342 (1992).
- Enoch, T., Carr, A. M. & Nurse, P. *Genes Dev.* **6**, 2035–2046 (1992).
- Enoch, T. & Nurse, P. *Cell* **60**, 665–673 (1990).
- Lundgren, K. *et al.* *Cell* **64**, 1111–1122 (1991).
- Rowley, R., Hudson, J. & Young, P. G. *Nature* **356**, 353–355 (1992).
- Painter, R. B. & Young, B. R. *Radiat. Res.* **64**, 648–656 (1975).
- Sorger, P. K. & Murray, A. W. *Nature* **355**, 365–368 (1992).
- Amon, A. *et al.* *Nature* **355**, 368–371 (1992).
- Weinert, T. A. *Radiat. Res.* **132**, 141–143 (1992).
- Weinert, T. A. & Hartwell, L. H. *Science* **241**, 317 (1988).
- Kuerbitz, S. J., Plunkett, B. S., Walsh, W. V. & Kastan, M. B. *Proc. natn. Acad. Sci. U.S.A.* **89**, 7491 (1992).
- Kastan, M. B. *et al.* *Cell* **71**, 587–597 (1992).

On the other hand . . .

Nigel A. Brown and Anthony Lander

IN one of those rare and remarkable papers announcing the completely unexpected, Yokoyama and colleagues¹ last month reported an insertional mutation that causes mice to develop with a body plan that is the mirror image of normal. For those intrigued by handedness this is a striking finding, not because of the reversed configuration itself, but because it occurs in all homozygous mutants. This has never been previously achieved, by experiment or nature (except perhaps in teleosts), and begs speculation on the molecular mechanism.

Vertebrates have numerous lateral asymmetries (stomach on the left, embryonic heart loops to the right and so on). Such asymmetries are generally extremely stable, both within and across species, but two classes of genetic variants in laterality are known: those of individual organs, and those of a random nature.

There are some species in which a particular trait has stable left and right versions which are genetically determined. In the development of flat fishes, for example, one eye migrates to the other side, and there are both left- and right-sided varieties of some species². And earlier this year, a predatory fish was described which has a mouth to one side and in which left- or right-mouthed development is apparently controlled by a single gene³. In these cases, however, the position of all other organs is normal.

There are several examples of 'randomization', that is an absence of handedness (but not asymmetry), so half the animals are normal, half completely inverted (known as *situs inversus*). These include mice with the *iv* mutation⁴ and

humans with primary ciliary dyskinesia, a syndrome which affects about 1 in 20,000 people and is a heterogeneous collection of autosomal recessive ciliary dysmotilities similarly associated with a randomized body plan⁵. Also, a number of treatments of rodent embryos in culture lead to *situs inversus*, but never with more than a 50 per cent incidence⁶.

It is easy to rationalize, at least theoretically, these kinds of genetically determined enantiomers. The usual explanation for an absence of handedness is that asymmetry is specified in development by an intrinsically random process which is biased by an asymmetrical signal (see ref. 7 for review). Loss of the signal results in randomization. By this argument, the mouse *iv* is a loss-of-function mutation of some component of the biasing mechanism. One can imagine several ways in which left and right traits might be produced in an otherwise normal (asymmetric) body plan. For instance the trait could be induced at a specific concentration range of an asymmetrically produced signal. All that would be required to switch from left to right would be a change in the amount of signal produced or in the sensitivity of the responding cells (see Fig. 1).

The new insertional mutant (*inv*)¹ was produced serendipitously in an attempt to create pigmented from albino mice by injecting a tyrosinase minigene into fertilized eggs. Several lines of transgenic mice were obtained, and when one of these was made homozygous for the inserted gene complete body inversion was observed in all animals. Because the other lines did not show this effect it is unlikely to be due to the minigene itself, but due to disruption of the host genome. Yokoyama *et al.* looked at *inv/inv* embryos and found inverted turning (Fig. 2) and heart looping.

Most would agree that handed positional information must be derived from a molecule or larger structure which is itself asymmetric

and, further, that to derive this information the molecule must be tethered with respect to the anteroposterior and dorso-ventral axes. For a mutation to cause 100 per cent inversion by a direct effect on this process, the molecule must either switch its asymmetry or the direction in which it is tethered. Neither seems very likely, especially for a loss-of-function mutation, which would be the expected result of an insertion. If the asymmetric molecule were a protein, it is possible that the native protein would tether in one direction, but a post-translational modification results in



FIG. 2 Normal (left in the picture) and mirror-image *situs inversus* (like *inv/inv*) 9.5-day-old mouse embryos. The turning process of wild-type embryos coils the tail to the right, while *inv/inv* embryos turn in the opposite direction placing the tail to the left.

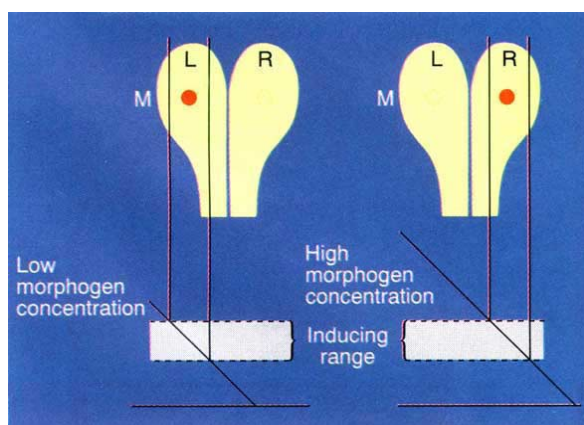


FIG. 1 A theoretical scheme for the genetic determination of a left or right structure, in the presence of a primary handed asymmetry. Both sides of the embryo are competent to develop into the structure, which is induced by a specific concentration range of a morphogen (M) produced from the left. Two alleles could result in low or high morphogen production, switching the structure from left to right.

opposite tethering. In this scheme, *inv* could be a loss-of-function of the post-translational modification.

Yokoyama *et al.*¹ themselves suggest that specification of asymmetry is a two-step procedure, in which a default pathway sets up asymmetry in one direction, and a second process switches this to the opposite direction. Thus, *inv* is a loss-of-function of the switch process. Formally, this is no different to a post-translational mechanism. Although it seems biologically wasteful to specify asymmetry in one direction only to reverse it, perhaps the main objection to the idea of a default inverted pathway is that it implies that a multistep process is involved, in which loss of function could quite easily lead to a completely inverted phenotype. But, as we have said, completely inverted phenotypes have never been observed before, in evolution or experiment.

Another possibility is that overall body plan asymmetry is normally specified by a mechanism like the one we suggested for an individual trait, in which both left and right forms can be derived from one primary asymmetry (Fig. 1). If this were the case, the effect of *inv* could simply be a change in morphogen concentration. The irony of this explanation is that, in fact, the primary asymmetry in *inv* would be quite normal.

Two further features of the *inv* mutants deserve mention. First, homozygous mice have kidney and liver defects, develop jaundice and die about a week after birth, problems which are unlikely to be a result of left-right indecision. It may be that a single gene has been disrupted and that it is involved in several developmental processes. This possibility would fit with a scheme in which *inv* is not specifically involved in left-right determination, but in a more general developmental event, and may explain the lack of other viable, fully inverted mutants. More likely, however, the insert may have disrupted more than one gene, perhaps inducing deletion or rearrangement, as is often the case (making the cloning of the disrupted genes much more difficult).

Second, animals homozygous for *inv* are not only inverted, but also have abnormalities of laterality: polysplenia (a marker of left isomerism, that is two left sides); heterotaxia (discordance of organ laterality within an individual); and heart defects. Such abnormalities are also found in all circumstances in which *situs inversus* is induced, be it genetic or environmental, in man or in mouse. The significance of

this is unclear, but it suggests that the polarity and fidelity of left-right specification are somehow linked.

The *inv* mutant provokes much thought, but it is not at all clear how it can be used in experimental embryology. The hope is that the insert will allow the cloning of the gene (or genes) that have been disrupted, and that this would lead to real advances. As usual, those interested in cerebral dominance will be waiting anxiously on the sidelines to see how many homologues of the gene they can find in the human genome. In the meantime, as Cosette says in *Les Misérables*, "Symmetry is ennui, and ennui is the very essence of grief and melancholy". □

Nigel A. Brown and Anthony Lander are in the MRC Experimental Embryology and Teratology Unit, St George's Hospital Medical School, London SW17 0RE, UK.

1. Yokoyama, T. *et al.* *Science* **260**, 679–682 (1993).
2. Policansky, D. *Sci. Am.* **246**, 96–102 (1982).
3. Hori, M. *Science* **260**, 216–219 (1993).
4. Layton, W. M. *J. Hered.* **67**, 336–338 (1976).
5. Moreno, A. & Murphy, E. A. *Am. J. med. Genet.* **8**, 305–313 (1981).
6. McCarthy, A., Wolpert, L. & Brown, N. A. *Teratology* **42**, 33A (1990).
7. Brown, N. A. & Wolpert, L. *Development* **109**, 1–9 (1990).

STAR FORMATION

A positive pregnancy test?

Anthony Whitworth

NEW stars are born all the time in interstellar space. The breeding grounds are giant molecular clouds, large aggregates of cold dense molecular gas, in the disks of spiral galaxies such as our own Milky Way. Of those that are near enough to have been observed in detail, all except the Maddalena cloud are giving birth to stars. Now J. P. Williams and L. Blitz (*Astrophys. J.* **405**, L75–L78; 1993) suggest that the Maddalena cloud is pregnant and will in due course also produce stellar offspring. If their suggestion is correct, the Maddalena cloud offers a unique and invaluable opportunity to study the conception and gestation of stars, and hence to understand what determines their final properties.

Giant molecular clouds have a complex internal structure, with a hierarchy of dense low-mass clumps and more diffuse high-mass clumps. This hierarchy ranges from clumps having masses less than that of the Sun (about 2×10^{30} kg) to those more than 3,000 times as massive. New stars condense out of the clumps because of self-gravity. The question is whether there is an evolutionary connection between clumps of different masses. Do smaller clumps agglomerate to form larger ones, or do larger clumps fragment? And under what circum-

stances do new stars condense? These questions are the key to understanding why star birth is prolific under some circumstances, and wholly absent under others.

Williams and Blitz have observed two

molecular clouds in line radiation from the molecule ^{13}CO , carbon monoxide in which the carbon atom is the rare isotope with an extra neutron. This is the most convenient method of observation as the radiation is produced by a rotational transition which is easily excited at the low temperatures in such clouds (less than about 30 K), and has a wavelength of 2.6 mm which does not suffer much from interstellar or atmospheric extinction. By observing line radiation, one can estimate radial velocity from the Doppler shift of the line centre, and thus separate clumps with different radial velocities that fall on the same line of sight. The rare isotopic form of CO is used because the signal is less likely to saturate, and therefore carries more information. The clouds cannot be observed in radiation from their principal constituent molecule H_2 , because it emits negligible radiation at low temperature.

Of the two molecular clouds that Williams and Blitz observe, the Rosette cloud is producing new stars, and in this respect it is broadly representative of giant molecular clouds in general. The second cloud, known as Maddalena after its discoverer, has no apparent evidence of ongoing star birth, making it unique amongst nearby (and hence well-observed) clouds. What Williams and Blitz assert is that the Maddalena cloud has more large and fewer small clumps than the Rosette cloud and than others for which the distribution of clump masses has been determined. They hypothesize that the paucity of low-mass clumps in Maddalena is symptomatic of an antenatal state; as Maddalena reaches full-term, the larger



David Malin/ROE

In the pink. The Rosette Nebula, illuminated from within by newly formed stars. Why is the Maddalena molecular cloud not similarly fecund?

clumps will tend to break up into smaller ones (possibly as a result of collisions), giving the clumps a mass distribution like that in other clouds. Williams and Blitz conclude that the internal structure of a giant molecular cloud evolves primarily by larger clumps fragmenting into smaller ones, and that agglomeration is unimportant.

Of course, in a field as fundamental as stellar obstetrics, such a view will not go unchallenged. For instance, it might be argued that the Rosette cloud contains many new stars, and that these heat their immediate surroundings to produce hotspots. Because CO emission is strengthened by both high density and high temperature, these hotspots would then masquerade as small clumps, thereby creating the illusion of an excess of small clumps.

Even if Williams and Blitz are right about the mass distribution of clumps, are we forced to conclude that clumps evolve primarily by fragmentation? In the Orion Nebula, star birth occurs almost exclusively in the large clumps (E. A. Lada, *Astrophys. J.* **393**, L25–L28; 1992), precisely those clumps that are overabundant in the Maddalena cloud. This suggests that the build-up to star birth involves the creation of large clumps, and not their destruction by fragmentation. Once star formation is underway, it will preferentially consume the large clumps. As most molecular clouds seem to produce several generations of stars in quick succession, the population of massive clumps will need to be replenished continuously. This could occur by agglomeration of smaller clumps.

In Williams and Blitz's scheme, molecular clouds are assembled first, and then predispose themselves to spawn stars by first adjusting their internal structure. If this scheme is correct, the Maddalena cloud may well be a crucial missing link in cloud evolution. However, there is an alternative scheme, in which the dynamical processes that assemble a molecular cloud are likely also to trigger star formation within the cloud, so that it comes into existence already forming stars. This would explain why the vast majority of molecular clouds are forming stars, and why the most prolific star formation occurs in the most dynamically disturbed clouds. In this scheme, the Maddalena cloud is just exceptionally quiescent, and thus more inhibited than others when it comes to forming stars. Whichever scheme is correct, it seems that the question of whether clumps evolve by fragmentation or agglomeration is still open. □

Anthony Whitworth is in the Department of Physics and Astronomy, University of Wales, Cardiff CF2 3YB, UK.

Springtime in the desert

Marvin Wickens

THE 3' end of messenger RNA in animal cells was long viewed as a desert — as barren, void of any important regulatory information, and carrying only the signals necessary to form the end of the mRNA and little else. Gradually, it has become clear that this perspective is misguided, particularly in early development. Between the translation termination codon and the poly (A) tail — in what is often a vast 3' untranslated region (3'UTR) — can lie signals which control an mRNA's translation, stability and location within the cell¹. Now, writing in *Cell*², Rastinejad and Blau go further. They show that signals in the 3'UTR can control not only the mRNA within which they reside, but other genes as well, and the results provide hints of networks of genetic interactions involving 3'UTRs.

These new developments began with an attempt to identify genes involved in muscle-cell differentiation. Cultured myoblasts turn on a variety of muscle-specific genes dramatically when growth is prevented, and then fuse and even form pulsating myotubes in a petri dish. To find genes involved in this process, Rastinejad and Blau first identified a mutant myoblast cell line that was unable to differentiate and fuse, and showed that it could not efficiently use a muscle-specific actin promoter. They then looked for genes that could complement the defect by introducing a library of cloned cDNAs from differentiating cells, selecting for expression from the actin promoter. Transfection experiments by others had shown that a few single genes, including those encoding MyoD, myogenin, MRF4 and myf5, could convert fibroblasts into myoblasts, implying that these genes might be master regulators of muscle differentiation³.

Stimulation

It turns out, however, that the DNAs isolated by Rastinejad and Blau are not new genes at the top of a ruling hierarchy, but humble 3'UTRs of perfectly orthodox muscle-specific mRNAs — those for actin, tropomyosin and tropomyosin I. Each of these three 3'UTRs can stimulate the level of mRNA from certain other muscle-specific genes *in trans*: express them at high levels, and the amount of RNA produced from actin and myogenin promoters increases.

These 3'UTRs could act either by stimulating transcription or by inhibiting cell growth. Proliferation and differentiation are antagonistic in myogenesis; for example, growth factors suppress differentiation, whereas their withdrawal

stimulates it⁴. Provocatively, the tropomyosin 3'UTR inhibits growth of non-muscle cells². Rastinejad and Blau reason that if the 3'UTRs also inhibited growth of myoblasts, that might be enough to trigger activation of certain muscle-specific genes. Although more direct effects on transcription are possible, it is striking that the action of the 3'UTRs is very different from that of the transcriptional regulators, like MyoD and myogenin. These proteins can turn on muscle-specific genes in fibroblasts; the muscle-specific 3'UTRs do not.

What sense does it make to have a product, made only in terminally differentiated cells, that stimulates differentiation? A cell containing tropomyosin mRNA presumably has already made the decision to differentiate, and to stop dividing. Why make the same decision again?

Key genetic decisions are often maintained by positive feedback loops. A little bit of λ repressor directs a bacterium to make more, stably keeping the virus quiescent and preventing lysis⁵; sex lethal protein in *Drosophila* enhances its own production by regulating splicing, and as a result reinforces the earlier decision to be female rather than male^{6,7}; similarly, several different myogenic transcription factors, including MyoD, may stimulate their own transcription (see for example ref. 8). What is more, they may also stimulate one another's transcription, forming a network of mutual reinforcement^{8–10}.

3'UTRs may be part of this same network: certain muscle-specific 3'UTRs may stimulate transcription not only of themselves but of other muscle-specific genes as well. In fact, one of the promoters activated by the 3'UTRs is that of the myogenin gene, which is itself a key regulator of muscle differentiation⁷. Through such an intricate plexus of positive feedback loops, the cell's decision to make muscle rather than to divide could be maintained and stabilized.

Whatever the logic of the circuitry, the question of how the 3'UTR of one mRNA affects another mRNA remains. 3'UTRs can control both translation and mRNA stability, presumably through the binding of specific proteins to specific RNA sequences. This being the case, excess 3'UTR segments, supplied *in trans*, might well sequester regulatory proteins away from the mRNAs they ought to be controlling. This kind of titration by 3'UTRs can occur *in vivo*. For example, the *fem-3* gene, a key mRNA for sex determination in worms, is regulated in part by its

3'UTR^{11,12}. Animals with excess copies of this 3'UTR appear to mis-regulate endogenous *fem-3* activity, and so differentiate aberrantly, presumably because the 3'UTRs tie up the regulatory RNA-binding protein. A simple enough picture.

To explain Rastinejad and Blau's results by such a factor titration model, an excess of one 3'UTR must cause mis-regulation not only of its own mRNA, but also of another. Most simply, the mRNA affected would be critical in the control of cell proliferation or transcription of muscle-specific genes. As yet, though, no myoblast mRNAs have been identified that respond to another's 3'UTR, either in translational activity or stability.

However, in other systems, hints of regulatory circuits involving 3'UTRs are beginning to emerge. The same protein binds to a stem-loop structure found in the 3'UTR of transferrin receptor mRNA, where it controls that mRNA's stability, and to an identical structure in the 5'UTR of ferritin mRNA, where it controls translation¹³. In flies, a short sequence in the 3'UTRs of *bicoid* and *hunchback* mRNAs apparently can repress their activity through the action of the regulatory protein, nanos^{14,15}. In mammalian cells, a variety of cytokine and growth-factor mRNAs contain repeats of the sequence AUUUA in their 3'UTRs, which help make these mRNAs very unstable¹⁶. In frog and mouse eggs, several mRNAs contain related U-rich sequences that cause them to receive poly (A) and become translationally active at specific times during early development¹⁷. It is possible (though unproven) that the same protein binds to the U-rich polyadenylation sequences of different mRNAs, or to all AUUUA-related instability elements.

Although titration of RNA-binding proteins is a simple way that 3'UTRs could act in *trans*, it is certainly not the only way. Cross-talk could be achieved by direct action of the 3'UTR on another mRNA either through base-pairing or ribozyme action.

Another issue is how the cell-culture results of Rastinejad and Blau bear on events in an intact animal. This is not straightforward: a myoblast differentiating in culture is not necessarily the same as muscle arising from somites in an embryo. Transgenic animals may help

here. But because several different 3'UTRs have comparable effects, elimination of any one of them in a transgenic animal may be of little consequence. Excess 3'UTR RNA in the differentiating myoblasts of a transgenic animal could cause aberrant myogenesis, although the effects of not properly reinforcing the decision to become muscle could be subtle; moreover, Rastinejad and Blau's data suggest that growth in some non-muscle lineages could also be inhibited.

Composites

Like the transcriptional control region upstream of a gene, 3'UTRs may be composites of regulatory elements and so provide opportunities for co-ordinated control. 3'UTRs, like promoters, can be large and relatively unconstrained during evolution. The only signals in the 3'UTR that must be preserved are six nucleotides required to form the 3' end of the mRNA¹⁸. No other region of the mRNA is so unconstrained: the sequence between the cap and the initiation codon must be scanned before translation begins, and secondary structure, bound proteins and even the length of the region can affect that process^{19,20}. The coding region has obvious and even more substantial restrictions. 3'UTRs may have become repositories for control elements precisely because they are out of the way and amenable to change.

Many of the first examples of regulatory elements in 3'UTRs involved maternal mRNAs¹. Indeed, it may turn out that regulation through 3'UTRs is less common in somatic cells than it is during very early development, before zygotic transcription begins. But do not be too sure. It is still early, and it is springtime in the desert. □

Marvin Wickens is in the Department of Biochemistry, University of Wisconsin, Madison, Wisconsin 53706, USA.

1. Wickens, M. (ed.) *Sem. dev. Biol.* **3**, 363–433 (1992).
2. Rastinejad, F. & Blau, H. M. *Cell* **72**, 903–917 (1993).
3. Weintraub, H. et al. *Science* **251**, 761–766 (1991).
4. Olson, E. N. *Dev. Biol.* **154**, 261–272 (1992).
5. Ptashne, M. *A Genetic Switch* (Blackwell Scientific, Oxford, 2nd edn, 1992).
6. Baker, B. *Nature* **340**, 521–524 (1989).
7. Bell, L. R. et al. *Cell* **55**, 1037–1046 (1988).
8. Thayer, M. J. et al. *Cell* **58**, 241–248 (1989).
9. Braun, T. et al. *EMBO J.* **8**, 3617–3625 (1989).
10. Edmondson, D. G. et al. *Molec. cell. Biol.* **12**, 3665–3677 (1992).
11. Barton, M. K., Schedl, T. B. & Kimble, J. *Genetics* **115**, 107–119 (1987).
12. Ahringer, J. & Kimble, J. *Nature* **349**, 346–348 (1991).
13. Klausner, R. D., Rouault, T. A. & Harford, J. B. *Cell* **72**, 19–28 (1993).
14. Wharton, R. P. & Struhl, G. *Cell* **67**, 955–967 (1991).
15. Gavis, E. R. & Lehmann, R. *Cell* **71**, 301–313 (1992).
16. Shaw, G. & Kamen, R. *Cell* **46**, 659–667 (1986).
17. Wickens, M. *Sem. dev. Biol.* **3**, 399–412 (1992).
18. Wickens, M. *Trends biochem. Sci.* **15**, 277–281 (1990).
19. Kozak, M. J. *Cell Biol.* **115**, 108, 229–241 (1989).
20. Kozak, M. *Gene Expr.* **1**, 117–127 (1992).

DAEDALUS

Sound unheard

THE hippopotamus, lucky creature, can close its ears. We cannot, though the human ear has a vestigial muscle for the purpose. We are defenceless against the steady stream of new and irritating noises that accompany every advance in technology. So Daedalus is inventing a volume control for ears.

Like any other microphone, the human ear reacts not to absolute pressure but to a pressure difference: that between the outside of the ear-drum and its inside. The inner ear is connected, via its eustachian tube, to the throat and nasal cavity. This is why artillery gunners open their mouths at the moment of firing. The sudden pressure-pulse on the outside of the ear-drum is countered by a pulse travelling up the eustachian tube and pressing on the inside, quietening the bang.

Daedalus's new 'nosephones' develop this elegant strategy. They launch a counter sound into the nostrils, and up the eustachian tubes to the inner ears. If the counter sound is an exact copy of the external sound, the ear-drums will never feel any difference of pressure. Their owner will be blissfully deaf to even the most insistent racket.

The prototype nosephone system fits onto a special hat. Twin microphones sample the sound close to the wearer's ears, and feed their sonic signals into two tubes inserted into his nostrils. He simply adjusts the intensity, phase and delay of the two nasal channels until the perceived sound is perfectly cancelled.

The commercial version will be miniaturized to deaf-aid proportions. Its unsightly nostril tubes will be replaced by internal, self-contained, induction-powered units. The happy user will revel in absolute control of his aural world. By turning the volume down, he will shut out all external sound while still hearing his own voice (a valuable trick for bores, and for public speakers facing a hostile audience). Conversely, a phase-inversion switch will let him turn the volume up. His ear-drums will then be pulled from within as well as pushed from without: useful for amplifying faint sounds. By adjusting the delay between left and right nostrils, he will be able to cancel sound from one specific direction (for example, the telephone or TV). And simply by switching off, he will recover his normal hearing. Nosephones can be worn as permanently as spectacles.

Yet noise can be addictive. For the children of the electronic age, silence is unnatural and abhorrent. So Daedalus is also inventing a cassette adapter, which cancels out all external sounds but generates an endless unsullied stream of internal pop-music. David Jones

Correction

OWING to an error in editing, a description of the isotope geochemistry of the East Pacific Rise near Easter Island in a News and Views article by Ken Rubin and John Mahoney (*Nature* **362**, 109–110; 1993) gave the impression that this was the sole work of Ken Rubin. In fact, the authors were reporting a collaborative effort of R. Poreda, J. G. Schilling, H. Craig, B. B. Hanan, D. Fontagnie and Rubin. □

Development of the zootype

SIR — I was delighted by the concept of the “zootype”¹ to describe animals by their common developmental pattern. Slack *et al.* define animals by zootype, the synapomorphy of Animalia; they tie this to Wolpert’s “positional information” concept (‘book’ and ‘map’) and to *Hox* genes. But there is a problem, exposed as the uniformity of the zootype in the multiplicity of phylotypic stages: if the interesting thing about the zootype is that everybody’s got one, why is it tied to just that stage which is so characteristically different in all animal groups?

There is an orthodox explanation, which is phase difference in the passage of information across the generations: maternal structures are required to ‘read out’ the zygotic genome. This was clearly seen by early embryologists. Conklin² put it well: we are chordates because our mothers were chordate, but we have blue eyes because of our own chromosomes. Many classical embryologists believed that egg architecture produced the organism’s basic structure, then mendelian genes added the details.

These classical ideas remain in today’s embryology. The late-blastula → gastrulation → phylotypic stage transfers morphogenetic control from mother’s organization of the oocyte (polarities, and many transcripts such as *bicoid*), to subsequent morphogenesis by the organized transcription of zygote genes. During this time the vast DNA information store (Wolpert’s ‘book’), like a magnetic tape, is set up in the differentiating embryo, laced properly into the tape player. DNA, like all information, needs context to be read out or expressed. This context (Wolpert’s ‘map’) is maternally provided gradients, ribosome machinery and messenger RNA transcripts with their labels, and constitutes the tape-player of my analogy. (The biochemical ‘mid-blastula’ transition is when zygote nuclear genes are first transcribed; but these are only effective later, further down the *Hox* cascade.)

Slack *et al.* found the idea of a phylotypic stage in the work of Reidel and Sander, but it goes back to Conklin and Wilson. In the early 1960s, I found the idea of an embryological concordance within each phylum so ‘obvious’ that I called it the “phyletic stage” in a textbook³. Sander and I agreed, in the late 1970s, that “phylotypic” was a better word, different from Haeckel’s discredited “phyletic”. However, my historical consideration of the idea⁴ was already published; I credited Sander with the change⁵.

What is the zootype, then, and what the phylotypic stage? The zootype exposes the most common method of set-

ting up animal development. Placozoa, Mesozoa and sponges are not animals, according to Slack *et al.*, if zootype is a definition of animal. However, these genuine animals might use wax cylinders, magnetic wire or vinyl disks while the rest of us use magnetic tape. Then the zootype idea works for all animals, and different phylotypic stages are different formats: chordates, annelids and coelenterates may be compared with cassette tapes, reel-to-reel and VHS, perhaps (were ammonites Betamax?). If the zootype genuinely is when Wolpert’s map is drawn — when the tape is threaded — then the phylotypic stage is the first music that is played, the theme of further development.

I believe this lyricism to be justified by the beautiful way in which Slack *et al.* have tied old and new embryologies together, and look forward to more biological laws of this kind.

Jack Cohen
39 Greenhill, Blackwell,
Bromsgrove,
Worcs B60 1BL, UK

SIR — Slack *et al.*¹ propose a particular pattern of expression of the set of homologous *Hox* genes as the defining character (synapomorphy) for the kingdom Animalia. The expression pattern of the *Hox* genes, which they name zootype, controls antero-posterior spatial relationships at a particular ontogenetic stage (the phylotypic stage) among a wide range of metazoan taxa. But their claim that this is the defining character for the Animalia is not substantiated. The phylotypic stage at which this pattern is expressed is indeed likely to be fundamental to animal evolution, but there are already other candidate characters for animal synapomorphy. The possession of collagen genes, for example, is clearly just as necessary for animal survival and morphogenesis^{6,7}. Systems that provide polarity during development will be important, but is the antero-posterior axis more fundamental than the dorso-ventral axis?

We can anticipate the discovery of similarly widespread mechanisms determining other morphological features. There are several animal synapomorphies, but Slack *et al.* give no evidence to suggest that their zootype represents the key innovation of animal development which allowed the rapid radiation of the group and might warrant its special status as “the defining character”. Indeed, it is unclear whether any morphological innovation is necessarily the key to the radiation of the Animalia. We believe a more fruitful approach would be to examine the relationships

among all potential synapomorphies cladistically, across a broad range of metazoan and non-metazoan taxa. Thus, which synapomorphies, if any, hold the special status of key innovations will be identified, and we may then understand what makes an animal an animal or, indeed, what makes an animal an iguana.

Nigel C. Hughes
Department of Paleobiology,
National Museum of Natural History,
Smithsonian Institution,
Washington, DC 20560, USA
Simon M. Hughes
MRC Muscle and Cell Motility Unit and
Developmental Biology Research
Centre,
King’s College London,
26–29 Drury Lane,
London WC2B 5RL, UK

SLACK *ET AL.* REPLY — We were aware of Cohen’s use of the term ‘phyletic stage’ but felt that, for vertebrates at least, he was considering a stage that was earlier than what we now regard as the phylotypic stage. He nominated the neurula as the phyletic stage of vertebrates and suggested that this was the first stage controlled by zygotic as opposed to maternal messenger RNA. We consider the neurula to be earlier than the phylotypic stage for three reasons: major morphogenetic movements are still going on; it does not yet display all major body parts as cell condensations; and it precedes the stage of maximum morphological resemblance between vertebrate classes. In invertebrate embryos as well, the onset of zygotic gene activity normally occurs well before the phylotypic stage.

Of course Cohen is right that there must be, at some point in the developmental programme, elements that connect the common plan of the zootype with the body plans of individual phyla. These will presumably lie among the genes controlled by the zootype genes, and we feel that the unravelling of these steps should now be high on the agenda of taxonomic research.

With regard to the Hughes’s point about collagen, we would not dispute that there may be other characters common to some or all animals, but we do think that the developmental programme is more fundamental than having one particular type of molecule. Because our

- Slack, J. M. W., Holland, P. W. H. & Graham, C. F. *Nature* **361**, 490–492 (1993).
- Conklin, E. G. *Heredity and Environment in the Development of Man* 184 (Princeton University Press, 1918).
- Cohen, J. *Living Embryos* (Pergamon, Oxford, 1963; 1967; 1981).
- Cohen, J. in *Maternal Effects in Development*, 4th Symp. Br. Soc. Dev. Biol. (eds Newth, D. R. & Balls, M.) 1–28 (Cambridge University Press, 1979).
- Cohen, J. in *Metamorphosis 8th Symp. Br. Soc. Dev. Biol.* (eds Balls, M. & Bownes, M.) 1–19 (Clarendon, Oxford, 1985).
- Adams, E. *Science* **202**, 591–598 (1978).
- Towe, K. M. *Life in the Universe* (ed. Billingham, J.) 297–306 (MIT, Cambridge, Massachusetts, 1981).

knowledge of the developmental programme outside *Drosophila* is fragmentary, we would obviously not claim that the zootype as we know it is complete. There are likely to be other conserved components and these will doubtless not be confined to the antero-posterior axis.

Finally, Peter Gilliver and Eleanor Lawrence have told us that we are not the first to use the term 'zootype'. In the *Oxford English Dictionary* (2nd edn, 1989) two previous usages are listed: in 1905 the *Daily Chronicle*, 4 September, referred to "Egyptian hieroglyphics and Totemic zootypes", and in 1897 the *Annual Report of the Smithsonian Institution* included the sentence "Out of this worm form type . . . all the higher ranges of zootypic evolution have sprung". We are grateful to know of these antecedents.

Jonathan Slack

Peter Holland

Chris Graham

Department of Zoology,
University of Oxford,
Oxford OX1 3PS, UK

Bateson and peacocks' tails

SIR — Enquist and Arak's paper¹ and the accompanying News and Views article by Howlett² about modelling the evolution of characteristics using a neural network are taken as qualified support for Darwin's sexual selection hypothesis. In the process, several problems emerge. First, the results are more consistent with the views of Bateson³ than of Darwin. Second, and consistent with Bateson's arguments, there is no justification for imagining that exaggerated male traits were selected. By this I do not mean to criticize sexual selection, or the competing hypotheses², *per se*. Rather, I wish to draw attention to the assumption that characters judged by us to be spectacular must have arisen by selection.

Bateson's views anticipated the theory of punctuated equilibrium, wherein dramatic and quantum changes can occur suddenly, providing the necessary variation for discontinuity between species. Whereas in Darwin's view variations were shaped over time by selective forces, Bateson believed that the physiology or genetics of organisms had the capacity to drive the observed discontinuity within a single generation. Enquist and Arak's work vindicates Bateson's views more than Darwin's. The provocative conclusions that "... the recognition mechanism itself exerts selection pressure on the signal. . ." and "that biases in the sensory apparatus . . . may have existed before the appearance of the same signals among males"¹ are

consistent with Bateson's contention that evolution is driven by the physiological variation of organisms themselves³.

Moreover, to argue that traits, such as a peacock's tail, are the products of selection is an inappropriate use of the evolutionary theory because it allows the generation of exclusive, yet unfalsifiable, hypotheses for the origin of traits³. Darwin's theory is appropriately used as evidence of the common descent of organisms. Its predictions are verified by the laboratory demonstration that variants are acted upon by natural selection^{4,5}. However, it does not provide a means for reconstructing the origins of any particular trait. The plethora of mechanisms described by Howlett demonstrates that reasonable people can construct equally reasonable alternative hypotheses based on the same data. This is because evolutionary theory is unidirectional: one can impose a selective force on a population to select variants but one cannot demonstrate that any given variation has been acted upon by natural selection.

To use the theory otherwise creates an argument that is circular: an 'important' trait must have been selected because selection acts on important traits. Furthermore, utility is not an explanation of a trait's origin. Taking away a sparrow's feathers to prevent flight might make the sparrow less 'fit', but that does not mean that the advantages provided by flight drove the evolution of feathers⁶.

Given the universality that the authors claim for their model of neural networks, bias should occur independently of sex. Therefore, outrageous traits should arise in both sexes, in all species, and in all network-controlled behaviours. Instead of a selection for deleterious characters, perhaps it is just as easy to imagine that "... animals live not only by virtue of, but also in spite of what [they] are"³. I am also troubled that the hypotheses offered to explain outrageous characteristics², and the experiment described in Fig. 3 of ref. 1, invoke successive modifications. Is there reason to believe that such a series of males actually existed? I do not wish to detract from the suggestion that neural networks may be internally biased¹. But although this may explain peculiar female preferences, it may be a mistake to attribute the origin of traits to sexual preferences.

Jack A. Heinemann

NIAID, NIH,
Rocky Mountain Laboratories,
Hamilton, Montana 59840, USA

SIR — With regard to Rory Howlett's News and Views piece about sexual selection², I wish to point out that the ornamental 'tail' feathers, each with its

iridescent eye pattern (shown in the accompanying photograph), are actually not part of the tail at all, but are the upper tail coverts. These remarkably elongated and elaborated feathers originate from the feather tract on the back, and create the stupendous 'train' that entirely hides the tail when the bird is at rest. The peacock's 'true' tail feathers are considerably shorter, dull-coloured, and otherwise uninteresting. In typical birds the tail coverts are short and unremarkable and only cover the base of the tail feathers.

For another sexually dimorphic pheasant, the great argus (*Argusianus argus*), it is the tail feathers and extremely modified secondary wing feathers that have evolved to great length and size, whereas the upper tail coverts are neither elongated nor specialized. This is an example of the reticulate nature of evolution, in which even closely related species have evolved, through sexual selection, oversized display ornaments from entirely distinct parts of the plumage.

Bruce M. Beehler

Wildlife Conservation Society and
Conservation International,
c/o Division of Birds MRC 116,
Smithsonian Institution,
Washington, DC 20560, USA

1. Enquist, M. & Arak, A. *Nature* **361**, 446–448 (1993).
2. Howlett, R. *Nature* **361**, 398–399 (1993).
3. Bateson, W. *Materials for the Study of Variation Treated with Especial Regard to Discontinuity in the Origin of Species* (Johns Hopkins University Press, Baltimore, 1992). (Originally published in 1894.)
4. Lederberg, J. *Genetics* **121**, 395–399 (1989).
5. Luria, S. E. & Delbruck, M. *Genetics* **28**, 491–511 (1943).
6. Gould, S. J. & Vrba, E. *Paleobiology* **8**, 4 (1982).

Delayed dispersal

SIR — J. Komdeur (*Nature* **358**, 493–495; 1992) reported that lifetime reproductive success (LRS) payoffs predicted the observed dispersal behaviour in a group-territorial warbler, and that birds chose when and where to disperse on this basis. I suggest that a null hypothesis that does not involve comparisons of habitat quality or LRS payoffs could also explain his observations. If birds survey only contiguous territories (the neighbourhood) for vacancies and contest for them when breeding opportunities appear, regardless of quality, the data interpreted by Komdeur as consistent with choice on the basis of anticipated LRS can be explained simply on the basis of reported mortality rates, the number of territories in the three qualities of habitat, and their location relative to each other.

As evidence of LRS-based dispersal, Komdeur stated, "Yearlings born on high-quality territories were more likely to remain at home as helpers (92.7%;

$n=41$) than those born on medium-quality territory (69.2%; $n=26$) or low-quality territory (28.6%; $n=56$). My suggestion will also account for this result because (1) nonbreeding competitors in a high-quality habitat are more numerous there because these territories produce more nonbreeders; and (2) vacancies in neighbourhoods of high-quality territories are rarer because of low annual mortality rates there (0.09) compared to medium (0.12) and low-quality territories (0.24), with corresponding life expectancies (7.4, 5.4 and 2.7 years in high, medium and low quality territories, respectively). Thus the queue of nonbreeders is longer in high-quality habitat.

As evidence of territory-quality based dispersal Komdeur claimed that birds hatched in high-quality territories were more likely to wait for high-quality territories than to accept a lower quality territory. But this could be explained merely by adopting the assumption of neighbourhood-restricted search. Similarly, Komdeur observed that 92.7% of breeders in low-quality territories came from low-quality territories – again as expected under the neighbourhood-search assumption. The lower number of origins of medium-territory-quality breeders from territories of the same quality rather than higher or lower quality can be explained by the much smaller number of medium-quality territories, a fact not explained by Komdeur's hypothesis. Komdeur further suggests that birds from high-quality territories are better competitors because 92.9% of vacancies in high-quality habitats were filled from high-quality territories. This result is expected, however, both from the assumption of neighbourhood search and from the larger number of nonbreeders produced in high-quality habitat. That nonbreeders from high-quality territories do not fill vacancies in low-quality habitat is expected on the assumption of neighbourhood search (1) from the easier access to low-quality vacancies from low-quality territories; and (2) from the observation that high-quality territories almost never border on low-quality habitat.

Jerram L. Brown

*Behavioral Ecology Group,
Department of Biological Science,
State University of New York,
Albany, New York 12222, USA*

Why microtubules grow and shrink

SIR — Maddox, in his article "Why microtubules grow and shrink"¹, misses the point. The paper he cites² does not answer this question, having more restricted intentions. Its logic is as follows: given that microtubules spontaneously grow and shrink, what are the consequences for the microtubule length distribution? This question was first treated numerically by Hill³. The fact that changes in tubulin concentration cause changes in dynamics is well established^{4,5}, and the concentration dependence has been studied experimentally⁶. Dogterom and Liebler² assume an empirical mathematical form for this dependence (see ref. 7) and solve for a one-dimensional length distribution function under conditions where tubulin concentration gradients are supported. Contrary to the impression given by Maddox, the possible mechanism underlying microtubule dynamic instability is not addressed by Dogterom and Liebler.

In fact, this mechanistic question has attracted quantitative biophysical study for a decade. The point is that a single microtubule, in the presence of a homogeneous concentration of tubulin-GTP, c_T , undergoes spontaneous transitions between periods of growth and shortening. The problem is to explain the existence of the transitions, and their quantitative properties. The transition frequencies depend on the value of c_T within a finite range spanning c_c , where c_c is the (critical) concentration at which the total mass of polymeric forms (microtubules) remains constant⁶. The justification for assuming homogeneity of concentration, and hence the applicability of the principles of chemical kinetics, is that for tubulin ($M_r=100,000$; measured lateral diffusion constant $D_{20,w}=5 \times 10^{-11} \text{ m}^2 \text{ s}^{-1}$), a concentration gradient imposed over dimensions $r=\pm 0.5 \mu\text{m}$ relaxes in about 10 ms ($t_{1/2} \approx r^2/D$). *In vitro* measurements show microtubules growing at $2\text{--}5 \mu\text{m min}^{-1}$, and shortening at $20 \mu\text{m min}^{-1}$ (not $20 \mu\text{m s}^{-1}$ as cited by Maddox); in 10 ms a microtubule end will have moved no more than $0.003 \mu\text{m}$ (in either growth or shortening). The diffusion of tubulin is much faster.

The underlying molecular mechanism of microtubule dynamic instability must address the different (chemical) kinetic properties of tubulin-GTP and tubulin-GDP (see refs 8, 9 for reviews). More important, the microtubule lattice structure, the kinetics of individual addition and dissociation steps, and the relationship between addition of tubulin-GTP to

the lattice and GTP hydrolysis, must be considered. Chen and Hill¹⁰ treated this system with Monte Carlo methods. Subsequently, there has been good experimental evidence that tubulin-GTP is present in dynamic microtubules only at, or below, the limits of analytical detection, which has necessitated a completely new treatment of the problem compared with that of Chen and Hill¹⁰. Quantitative numerical modelling of dynamic instability which incorporates this feature, and which rationalizes the transitions between microtubule growth and shortening in terms of a single terminal layer of tubulin-GTP at the microtubule end, has recently been reviewed¹¹.

Peter Bayley

*National Institute for Medical Research,
Mill Hill,
London NW7 1AA, UK*

1. Maddox, J. *Nature* **362**, 201 (1993).
2. Dogterom, M. & Liebler, S. *Phys. Rev. Lett.* **70**, 1347–1350 (1993).
3. Hill, T. L. *Proc. natn. Acad. Sci. U.S.A.* **81**, 6728–6732 (1984).
4. Mitchison, T. J. & Kirschner, M. W. *Nature* **312**, 232–237 and 237–242 (1984).
5. Horio, T. & Hotani, H. *Nature* **321**, 605–607 (1986).
6. Walker, R. A. et al. *J. Cell Biol.* **107**, 1437–1448 (1988).
7. Chen, Y. D. & Hill, T. L. *Proc. natn. Acad. Sci. U.S.A.* **84**, 8419–8423 (1987).
8. Gelfand, V. & Bershadsky, A. D. *A. Rev. Cell Biol.* **7**, 93–116 (1991).
9. Erickson, H. P. & O'Brien, E. T. *A. Rev. biophys. Biomolec. Str.* **21**, 145–166 (1992).
10. Chen, Y. D. & Hill, T. L. *Proc. natn. Acad. Sci. U.S.A.* **82**, 1131–1135 (1985).
11. Bayley, P. M. & Martin, S. R. *Comments Theor. Biol.* **2**, 403–427 (1992).

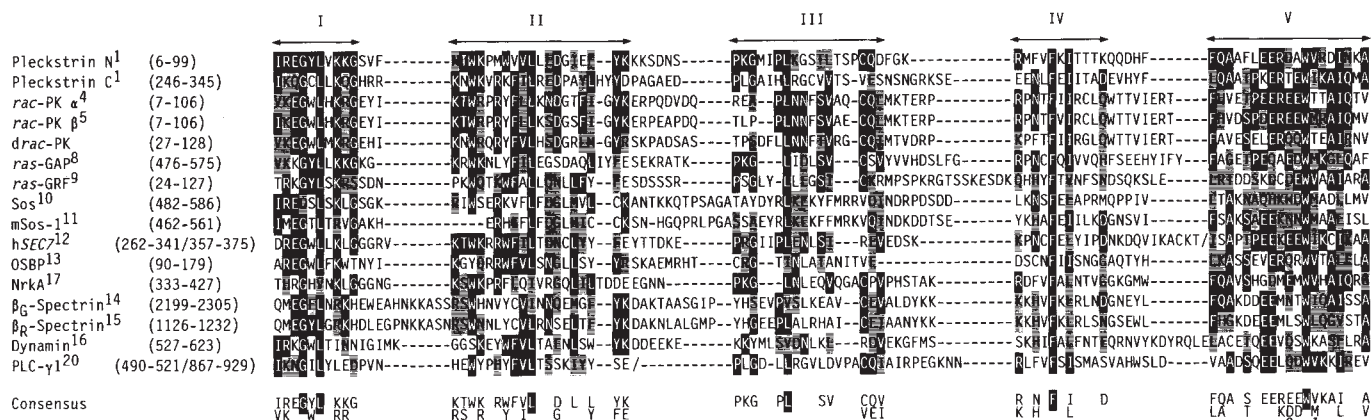
Pleckstrin domain homology

SIR — The major protein kinase C substrate of platelets, pleckstrin, contains N- and C-terminal domains each of about 100 residues in length (pleckstrin N and C), that have similar amino-acid sequences¹. We have recently become aware of an increasing number of proteins important in signal transduction or in cytoskeletal function that contain related sequences (see figure). This suggests that there is a pleckstrin homology (PH) domain, comparable in many respects with SH2 and SH3 domains^{2,3}. Indeed, the proteins with high similarity scores with respect to both pleckstrin domains include the *rac* (refs 4, 5) or *akt* (ref. 6) protein kinases, which at one point were thought to contain an SH2-like domain⁶. However, the crystal structure of the v-src SH2 domain has indicated that this is not the case⁷, and our present analysis shows that this domain is an example of a PH domain and has an entirely distinct consensus sequence.

Other proteins containing a PH domain include *ras*-GAP (ref. 8), *ras*-GRF (ref. 9) and the products of the *Drosophila* *Son of sevenless* gene (*Sos*)¹⁰ and its mouse hom-

Scientific Correspondence

Scientific Correspondence is intended to provide a forum in which readers may raise points of a scientific character. They need not arise out of anything published in *Nature*. In any case, priority will be given to letters of fewer than 500 words and five references.



Alignment of protein sequences similar to the N- or C-terminal domains of pleckstrin. *drac*-PK, *Drosophila rac* protein kinase, which has recently been cloned (B. A. H., manuscript in preparation). Pairwise similarity scores were determined and evaluated using the FASTA program RDF2 (ref. 18); the protein domains shown had initial or optimal scores greater than 6 s.d. above the mean from 200

shufflings of the N or C-terminal domain of pleckstrin ($z > 6$). The amino acid residues occurring most frequently in aligned positions are shown as white on black and replacements with positive scores in the PAM 250 matrix as black on grey. The consensus sequence, including alternatives, is shown below; the most highly conserved residues which anchor the subdomains are highlighted.

ologue (*mSos*-1)¹¹, all of which interact with *ras* proteins. Two other proteins with possible roles in signal transduction that contain a PH domain are the product of a human homologue of yeast *SEC7* (*hSEC7*)¹² and oxysterol-binding protein (*OSBP*)¹³. Although the PH domains of most of the above proteins show only slightly different similarity scores when compared with the two pleckstrin domains, those of *ras*-GAP and *Sos* are more closely related to pleckstrin C than to pleckstrin N. By contrast, PH domains that much more closely resemble pleckstrin N are found in the non-erythroid β _G- and 270K β _R-spectrins^{14,15}, in *dynamin*¹⁶ and in the C-terminal regulatory domain of a recently cloned trypanosomal protein kinase, *NrkA* (ref. 17). Analysis using FASTA (ref. 18) shows that the most significant relationships ($z > 15$) were between pleckstrin N and either *ras*-GRF or *NrkA* and between pleckstrin C and *rac*-PK β , *ras*-GAP or *hSEC7*. The discovery of a trypanosomal PH domain suggests that this sequence is of distant evolutionary origin and may have diverged to yield variants that interact with different members of the same protein family or with distinct but related protein motifs. Pleckstrin is likely to interact with two such proteins or motifs. This situation is comparable with that in many signal transduction proteins containing SH2 and SH3 domains, which often include two domains of the same type^{2,3}. Interestingly, some proteins with SH2 and/or SH3 domains, such as *ras*-GAP, also contain a PH domain. Another example (drawn to our attention by B. J. Mayer) is the growth factor receptor-bound protein, GRB-7 (ref. 19), which contains a poorly conserved PH domain that nevertheless has significant similarity to the PH domains of 270K β _R-spectrin, *hSEC7* and *ras*-GAP.

Comparison of the PH domains of the above proteins shows that, as with the

SH2 domain², five conserved subdomains can be distinguished. Although subdomains I and II are, in most instances, separated by two to four residues, the non-erythroid β -spectrins possess 11-residue inserts in this region, whereas subdomains IV and V are separated by an exceptionally long insert in *hSEC7*. Subdomain III is the least well-conserved of the five. An important question is the extent to which these subdomains can function independently. In this connection, we have discovered that the sequence of phospholipase C- γ 1 (for example, ref. 20) contains a PH domain that is split between subdomains II and III by insertion of the SH2-SH2-SH3 domain. Indeed, this PH domain largely fills the gaps between the SH2-SH2-SH3 region and the surrounding X and Y catalytic domains. By contrast, the δ -isozymes of phospholipase C contain intact but slightly modified PH domains in their N-terminal regions.

We speculate that phosphorylation of pleckstrin may regulate the interactions of its PH domains with other proteins and that these domains may be effectors in pathways of signal transduction leading to secretion of platelet granule constituents. By contrast, the PH domains present in known enzymes seem more likely to be involved in the input than the output of regulatory information. A detailed study of the effects of deletion of PH domains and of the interactions of PH domains with other proteins is needed to resolve this apparent conflict. The presence of PH domains in several proteins that interact with *ras* proteins raises the possibility that this domain is involved in the regulation of low *M_r* GTP-binding proteins. Finally, the PH domains of non-erythroid β -spectrins appear to complement the SH3 domains of the α -spectrins³, and similarly suggest a role in signal transduction to or from the membrane cytoskeleton. Our identifica-

tion of the PH domain as a novel motif likely to participate in the protein-protein interactions involved in signal transduction provides a new focus for further experiments. In particular, molecular biological and biochemical techniques can now be used to determine the functions of known PH domains and to identify new proteins with this domain.

Richard J. Haslam*

Hiroshi B. Koide†

Departments of Pathology* and

Biochemistry*†,

McMaster University,

1200 Main Street West, Hamilton,

Ontario, Canada L8N 3Z5

Brian A. Hemmings

Friedrich Miescher-Institut,

PO Box 2543,

CH-4002 Basel, Switzerland

1. Tyers, M. *et al.* *Nature* **333**, 470-473 (1988).
2. Koch, C. A., Anderson, D., Moran, M. F., Ellis, C. & Pawson, T. *Science* **252**, 668-674 (1991).
3. Musacchio, A., Gibson, T., Lehto, V.-P. & Saraste, M. *FEBS Lett.* **307**, 55-61 (1992).
4. Jones, P. F., Jakubowicz, T., Pitossi, F. J., Maurer, F. & Hemmings, B. A. *Proc. natn. Acad. Sci. U.S.A.* **88**, 4171-4175 (1991).
5. Jones, P. F., Jakubowicz, T. & Hemmings, B. A. *Cell Reg.* **2**, 1001-1009 (1991).
6. Bellacosa, A., Testa, J. R., Staaf, S. P. & Tsichlis, P. N. *Science* **254**, 274-277 (1991).
7. Waksman, G. *et al.* *Nature* **358**, 646-653 (1992).
8. Trahey, M. *et al.* *Science* **242**, 1697-1700 (1988).
9. Shou, C., Farnsworth, C. L., Neel, B. G. & Feig, L. A. *Nature* **358**, 351-354 (1992).
10. Simon, M. A., Bowtell, D. L., Dodson, G. S., Laverty, T. R. & Rubin, G. M. *Cell* **67**, 701-716 (1991).
11. Bowtell, D., Fu, P., Simon, M. & Senior, P. *Proc. natn. Acad. Sci. U.S.A.* **89**, 6511-6515 (1992).
12. Liu, L. & Pohajdak, B. *Biochim. biophys. Acta* **1132**, 75-78 (1992).
13. Levanon, D. *et al.* *Genomics* **7**, 65-74 (1990).
14. Hu, R.-J., Watanabe, M. & Bennett, V. *J. biol. Chem.* **267**, 18715-18722 (1992).
15. Winkelmann, J. C., Costa, F. F., Linzie, B. L. & Forget, B. G. *J. biol. Chem.* **265**, 20449-20454 (1990).
16. Obar, R. A., Collins, C. A., Hammarback, J. A., Shpetner, H. S. & Vallee, R. B. *Nature* **347**, 256-261 (1990).
17. Gale, M. & Parsons, M. *Molec. biochem. Parasit.* (in the press).
18. Pearson, W. R. *Meth. Enzym.* **183**, 63-98 (1990).
19. Margolis, B. *et al.* *Proc. natn. Acad. Sci. U.S.A.* **89**, 8894-8898 (1992).
20. Burgess, W. H. *et al.* *Molec. cell. Biol.* **10**, 4770-4777 (1990).

Atomic conspiracies

David Cassidy

Heisenberg's War: The Secret History of the German Bomb. By Thomas Powers. Knopf/Cape: 1993. Pp. 610. \$27.50, £20.

THIS book attempts to answer a question that has been hotly debated for decades: why didn't Germany produce an atomic bomb during the Second World War? Unfortunately, the author is so superficial in his approach and so prejudiced in his handling of sources as to render his account, although closely argued, quite unconvincing.

Since the end of the war, the debate has gradually turned to the historical documents, in addition to the recollections of the participants. The volume and accessibility of these sources have increased almost exponentially over the years. Transcripts of conversations among ten leading German scientists during their six-month captivity (beginning in July 1945) at Farm Hall in the United Kingdom were released last year and are now in the press (*Operation 'Epsilon': The Farm Hall Transcripts*, IOP/University of California Press). Historical scholarship has intensified accordingly, resulting in two recent attempts to attain a well-founded comprehension of the German nuclear programme: Mark Walker's *German National Socialism and the Quest for Nuclear Power, 1939-1949* (CUP, 1989) and my biography of Werner Heisenberg, *Uncertainty* (Freeman, 1992).

Powers ignores this work because he is in complete disagreement with it. He also largely ignores the "vast paper record . . . which has mainly occupied historians of the [German] effort". When he does consult these documents, his use of them is highly questionable, for he is providing "another kind of history . . . a shadow history of what German scientists thought, felt and said to each other in the small hours of the night about the work they had been given to do."

Statements

Where is this 'shadow history' to be found? Despite mention of letters and diaries, Powers seeks it in the post-war statements of Heisenberg himself and his closest collaborator, C. F. von Weizsäcker. But, according to Powers, even reliance on Heisenberg can go only so far: "We may build our case for answers as tightly as we like, but what we say remains hostage to what Heisenberg was willing to concede, and questions of motive and intention cannot be established more clearly than he was willing to state them."

History held 'hostage' to its subject is no history at all; research that grasps at shadow realities while ignoring the written record is as unacceptable in history as are appeals to the occult in modern science. What's more, Powers is remarkably selective in his reliance on memory. For instance, he allows that Niels Bohr's impression of a meeting with Heisenberg in 1941 "altered subtly with the changes in Bohr's situation and the passage of time"; in the case of Bohr and S. A. Goudsmit, he even goes so far as to say that, because of the emotional climate, "truth-telling ended soon after 1945". Apparently, only Heisenberg and von Weizsäcker are to be believed.

So why didn't Germany produce a bomb? As with Robert Jungk before him (*Brighter than a Thousand Suns*, Harcourt Brace Jovanovich, 1958), the answer for Powers is clear: Heisenberg sabotaged the effort. Briefly, Powers's argument is this: Heisenberg quickly became the controlling figure in German fission research. When he, von Weizsäcker and F. Houtermans learned in 1941 that a reactor would breed fissionable material (later called plutonium), they conspired to hide the implications, to delay further research and to alert the Allies that they would not build a bomb. A message relayed to Washington third hand in April 1941 stated that the Germans were striving for an atom bomb but that "Heisenberg himself tries to delay the work as much as possible." Powers claims that in September 1941 Heisenberg went to Copenhagen to give a similar message to Bohr, which Bohr accepted. When Bohr escaped from Denmark, says Powers, he became convinced that Heisenberg had duped him and was, after all, working on a bomb. A suggestion by two *émigré* scientists led, claims Powers, to a plot to assassinate Heisenberg should he (Heisenberg) reveal work on a bomb (he did not) during his visit to Switzerland in 1944.

Meanwhile, as German authorities learned that plutonium could be used as an alternative to uranium in nuclear explosives, Heisenberg directed the entire German nuclear project "into a broom closet" by encouraging a modest amount of research into reactor development while discouraging optimism about a bomb. Powers claims that Heisenberg mentioned plutonium only briefly in a speech to authorities in February 1942 and not at all to A. Speer, the German

Minister of Munitions, in June. Powers asserts that, later at Farm Hall, Heisenberg revealed a crucial piece of evidence: that he had already made a correct calculation of the critical mass of uranium-235, which indicated that a bomb could be made more easily than anyone else had realized. He had kept this secret to himself. At the same time, Allied scientists, unable "to accept as a fact" that he was not determined to build a bomb, refused to accept his claimed reticence and its apparent ethical origins. According to Powers, they instead attributed the failure to build a bomb to scientific errors and to the decline of German science under Nazi dictatorship. Some, feeling guilty about the assassination plot, came to Heisenberg's defence, but Heisenberg soon gave up the attempt to convince others about his efforts to delay the work.

This may make a good story, but as history it is incredible. I can touch here only briefly on a few key points.

Assumptions

First, Powers's account hinges on the assumption that Heisenberg alone controlled the German nuclear project. It is true, as Powers states, that scientists do control judgements about technical feasibility, but records show that Heisenberg was politically suspect until as late as June 1942 and that other scientists closer to the Nazi regime were equally influential. The records also indicate that these scientists provided the German army with a report in December 1941 urging it to sponsor fission research — with mention of plutonium; but because of limited resources, not Heisenberg's reticence, the army curtailed its support.

Second, Heisenberg himself stated repeatedly after the war that scientists were spared the moral decision of whether to build an atomic bomb. At Farm Hall, he repudiated von Weizsäcker's suggestion that moral scruples had prevented the Germans from building an atomic bomb. Max von Laue, in a letter quoted by Powers, states that the Farm Hall scruples were just a *Hesart* (version) of history: "I did not hear the mention of any ethical point of view." Heisenberg's main concern for years had been the preservation of German science, not scruples, and wartime nuclear research provided a new opportunity to pursue this goal. Despite the message to Washington in April 1941, there is no indication that Heisenberg delayed nuclear research during 1941 and 1942, although he did express relief in the end that it did not lead to an atomic bomb. Rather than dragging his feet, he worked intensively on two nuclear projects, in Leipzig and Berlin, until June 1942, even neglecting his family and his personal research to do so.

Third, far from playing down the importance of plutonium in his speech in February 1942 to Hitler's officials, Heisenberg mentions the subject prominently (*Collected Works of Werner Heisenberg*, eds W. Blum *et al.*, Springer, 1989). He later reminds his audience that a reactor by-product will be explosive and that uranium-235 is also an explosive "of unimaginable strength". If he fell silent to Speer, it was only for fear of being ordered to produce the new weapon on demand.

Fourth, if Heisenberg really did know that the critical mass for a uranium bomb was as small as it is, then why was he so shocked when he learned of Hiroshima? Why was his calculation of the critical mass so flawed in his lecture to his fellow internees at Farm Hall on 14 August 1945 (see J. Bernstein, *New York Review of Books* 39, 47–53; 13 August 1992 and J. L. Logan & R. Serber, *Nature* 362, 117; 11 March 1993)? And why did he tell Otto Hahn on 6 August that "quite honestly I have never worked it out as I never believed one could get pure '235'"?

Finally, there is no credible evidence for the assassination plot, nor is it plausible.

The Allies knew Heisenberg was working on fission: why not assassinate him outright, instead of waiting for him to reveal his research to a foreign audience? Why do the deed in a public forum? Why select an agent as unlikely as a multilingual baseball player? And why not target other travelling German scientists?

In rejecting Powers's portrayal of Heisenberg as hero, I do not mean to imply that Heisenberg was a fiendish villain, bent on producing nuclear weapons for Hitler or for Germany. Rather, he was what we might expect: a highly talented, cultured individual of normal decency who was unfortunately caught up in the dreadful circumstances of his time for which he, like most people, was totally unprepared. At times he did use his position to worthy ends. But to argue beyond that — to claim that he alone acted so very differently from the rest — is to stretch credulity, and the historical record, beyond the breaking point. □

David Cassidy is in the Natural Science Program, Hofstra University, Hempstead, New York 11550, USA.

devotes nearly 40 pages to the marine carbon cycle; it takes a dedicated reader to wade through all the details. In between are chapters such as that on atmospheric chemistry that fully realize the goal of the volume.

Similar disparities are found in the modelling chapters, partly influenced by the authors' desire not to duplicate too much of what appears in the background science sections. The chapter on sea ice modelling presents many of the detailed equations; the chapter on land ice modelling has almost no equations; and the discussion of atmospheric chemistry modelling spends almost all of its time on numerical solutions to the transport equations. There is also a varying tone in the authors' assessments of how well the individual models can simulate reality, ranging from brutal honesty to Pollyanna myopia. Again, occasional chapters stand out, such as the accessible and comprehensive one on ocean modelling.

How is the field represented by this book? There is some parochialism in the choice of contributors; more than half of the chapters are written by scientists at the National Center for Atmospheric Research in Boulder, Colorado, or at nearby organizations, and so some points of view are neglected. A more serious problem, however, arises because most of the modelling is done by physicists and numerical analysts. Rarely, for example, are biologists fully involved in modelling the biospheric components, chemists in modelling atmospheric or ocean chemistry, or oceanographers in producing global ocean models. (Oceanographers usually emphasize the importance of mesoscale eddies; the ocean modelling chapter, however, focuses on models that omit these eddies.) Specialists in these fields often rebel vociferously against the approaches of climate system modellers, and consider the models to be simplistic and misleading. I once had occasion to show a well-known land surface model to a prominent soil scientist. After reading the equations, he commented: "This is how I would have thought it worked if I didn't know better."

Is climate system modelling the ultimate example of hubris, or, by chopping away at areas of ignorance, will we truly improve our predictive capability? A thorough reading of *Climate System Modeling* provides support for both points of view. Unfortunately, our newly found capacity to alter the climate system through increases in carbon dioxide and so on makes this more than simply an academic question. □

David Rind is in the NASA Goddard Space Flight Center, Goddard Institute for Space Studies, 2880 Broadway, New York, New York 10025, USA.

A man's reach must exceed his grasp . . .

David Rind

Climate System Modeling. Edited by K. E. Trenberth. Cambridge University Press: 1993. Pp. 788. £35, \$49.95.

To model the climate system, we apparently have to understand (with apologies to Douglas Adams), "life, the universe and everything". For example, in addition to knowledge about the atmosphere and climate, we must know how vegetation responds to soil moisture deficits (which affects the severity of droughts) and how marine biota take up nutrients in a food web (which affects the ocean's ability to absorb carbon dioxide). To understand the climate system's natural variability we must be able to quantify past and present climate forcing, which includes learning more about solar variability and stellar evolution. The concluding paragraph of this hefty tome rightly notes that "numerical modeling of the climate system is . . . one of the grand scientific challenges of our time"; and before the reader is finished with this survey of topics to be understood and modelled, he or she will be convinced that we need to know just about everything.

The book was conceived as a way for graduate students specializing in one

area of the climate system to learn about the issues and techniques in modelling other, often adjoining areas. It should serve equally well for their professors. Chapters first present the science underlying the various subsystems, including the atmosphere, ocean, land surface, terrestrial biology, atmospheric chemistry and marine biogeochemistry. Then models of these components are introduced, in greater or lesser detail. The writing is of uniformly high quality by specialists in each field; the figures are attractive and of uniform style; and considerable effort has been made to cross-reference concepts among the different chapters.

Can climate system modelling really be learned this way? Readers will undoubtedly pick up some information about each speciality, although the presentations differ markedly in their approach. The chapter on atmospheric science tries to cover the whole field in 60 pages, and in some depth too. It is hard to imagine that a student unfamiliar with most of the concepts will be able to digest all of this concentrated material. In contrast, other authors discuss only the portion of their discipline that relates to the climate system. The marine biogeochemistry chapter, for example,

Arid landscapes

Ken Pye

Desert Geomorphology. By Ron Cooke, Andrew Warren and Andrew Goudie. UCL Press: 1993. Pp. 536. £75, \$125 (hbk), £24.95, \$59.95 (pbk).

ALTHOUGH deserts cover about one-third of the Earth's land surface, only in the past few decades have they received their fair share of attention from Earth scientists. Desert research has recently been spurred on by the need to understand hydrocarbon reservoirs and surface conditions on other planets such as Mars, by concern about the effects of both natural and human-induced environmental change in drylands, and by the development of new technologies such as remote sensing.

The first synthesis of warm desert processes and landforms was Ron Cooke and Andrew Warren's *Geomorphology in Deserts* (Batsford, 1973), which became something of a classic. It is therefore appropriate that these authors have combined forces with Andrew Goudie to produce a new text that updates and extends the scope of its predecessor. The book combines an extensive review of the literature (the reference list contains some 2,000 entries) with examples drawn from the authors' own experiences in many of the world's deserts, including those of North and South America, the Middle East, Africa and the Indian subcontinent.

The five sections cover introductory global perspectives, desert surface conditions, fluvial processes and landforms, aeolian processes and landforms, and desert landform development. The ap-

proach is traditionally geomorphological, focusing on the nature of the Earth's surface processes and their relationship to landform development. Treatment of aspects such as weathering and the development of stone pavements, alluvial fans and dunes is thorough and original, but little attention is given to basin-scale and long-term controlling factors and interrelationships. Tectonic and structural controls on processes, sediment production, and landform evolution and preservation are given scant attention, and there is limited discussion of subsurface hydrology and arid-zone lakes. Geomorphological evidence of climate change in warm deserts is reviewed and the history of the main deserts is outlined, but there is surprisingly little explicit discussion of the causes and mechanisms of environmental change in deserts. Equally surprising is the limited attention given to the use of geomorphological principles in desert environmental management.

The writing of a book with such breadth in a fast-moving field is an ambitious task, with material rapidly becoming out of date. Much of this text has the feel of a late 1989 vintage, with minor later additions. Nevertheless, *Desert Geomorphology* provides an excellent synthesis for undergraduate and postgraduate students in geomorphology and other branches of Earth science. It should also be an essential purchase for seasoned researchers and practitioners, for whom it will provide a useful source of background information and intellectual nuggets. □

Ken Pye is in the Postgraduate Research Institute for Sedimentology, University of Reading, Whiteknights, Reading RG6 2AB, UK.

Theories and controversies

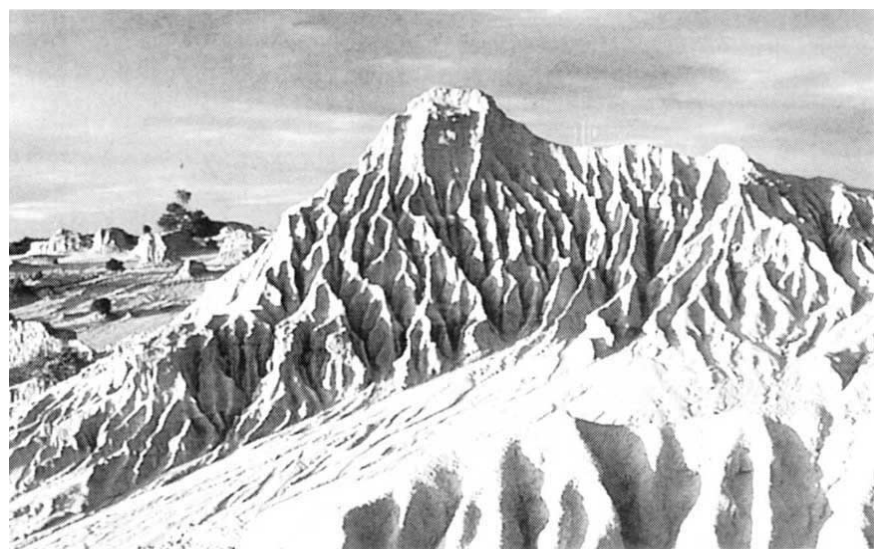
Christopher Wills

Evolution. By Mark Ridley. Blackwell Scientific: 1993. Pp. 670. £39.50, \$39.95 (hbk), £19.50 (pbk).

GOOD textbooks shape a field. They present up-to-date information in such a well-organized way that gaps in our knowledge become glaringly apparent. Alert students who read such books are driven to ask how these gaps might be filled in, and sometimes to ask even more fundamental questions about the field. The late Albert Lehninger's *Biochemistry* was such a text, as were James Watson's *Molecular Biology of the Gene* and E. O. Wilson's *Sociobiology*. I expect that Mark Ridley's new book on evolution will join their distinguished company.

Students who already have a good background in the biological sciences will get the most out of Ridley's book, which does not try to do everything. It is not an introductory text like M. W. Strickberger's *Evolution* (Jones and Bartlett, 1990), and it goes into more detail and more subjects than D. J. Futuyma's widely used *Evolutionary Biology* (Sinauer, 1986). The book is not heavily freighted with examples, and the examples that are used are highly selective, leading into complex and well-balanced discussions of evolutionary theories and controversies. This is the book's great strength.

Like Dante's Virgil, Ridley guides the well-prepared student through the many conflicting points of view that make evolutionary biology so daunting for the beginner. Calmly and at length, he weighs the various merits and demerits of nature versus nurture, Darwinism versus Lamarckism, group, individual, hard and soft selection. Along the way he adjudicates in the war between neutralists and selectionists, the three-way battle among cladists, phylogeneticists and evolutionary taxonomists, the 'slug-fest' between gradualists and punctuationalists, and the quarrel among sympatric, parapatric and allopatric speciationists. There is even a glance at creationists, frozen in the ice of the lowest level. Their views are given more attention than I suspect they deserve, which may be due to the fact that Ridley teaches in a state (Georgia) that actually adopted a creationist biology textbook. Be that as it may, he patiently examines the logic underlying evolution, transformism and creationism, and his arguments for evolution as the most likely explanation for the diversity of life should convince



Ancient silty dunes, now dissected by water erosion, form the 'Walls of China' near the dry Lake Mungo in southeastern Australia. Taken from the well-illustrated and comprehensive *Deserts: The Encroaching Wilderness* edited by T. Allan and A. Warren. Oxford University Press/Mitchell Beazley, \$35, £19.99.

the most benighted backwoodsman.

Perhaps the most impressive thing about the book is that the author takes nothing for granted. Each chapter builds a careful edifice of logic, examining the underpinnings of a particular aspect of evolutionary theory. Along the way, there are some very fine set-pieces. The section on population genetics is excellent, with the finest and clearest discussions of migration and multilocus genetics that I have seen in an introductory text. Ridley also has sensible things to say about the problem of species, carefully presenting ideas underlying the biological, phenetic, recognition and ecological species concepts and showing why they all provide necessary parts of the puzzle.

I particularly enjoyed his discussion of punctuatedism, which is carefully and appropriately embedded in a much larger general examination of evolution rates, macroevolutionary trends and patterns of extinction. These sections bring a welcome sense of balance to this needlessly polarized debate. And he gives a careful analysis of a debate that is at the very core of our understanding of how evolution works, the conflicting views on how genetic variation is retained in populations in the face of long-continued stabilizing selection. R. Lande's contention that newly arising mutation is enough to supply the variation that is lost is contrasted with M. Turelli's argument that unrealistically large numbers of mutations of large effect would have to take place. The only thing I missed in this discussion was an examination of the question of whether most variation found in natural populations really is in fact newly arisen. This leads to the further question of whether and how the phenotypic variation produced by new mutations can be distinguished from similar phenotypic variation that is produced by mutations with a longer selective history. But, because the various arguments are set out so clearly, students reading this section should be led to wonder about these questions and about the possibility of designing experiments to address them.

There are, of course, a few things that are left out or not dealt with in as much depth as they deserve. The most obvious area of contention that Ridley misses is that surrounding the origin of life, and there is a disappointingly brief discussion of the origin of the genetic code. Generally, molecular evolution is dealt with rather briefly and in several scattered places throughout the book. Discussions of the roles of transposable elements and viruses in mutation turn up here and there, but not in the logical place, the chapter on mutation. The discussion of mitochondrial evolution is rather out of date. I was disappointed that the seminal

work of T. Dobzhansky on chromosomal polymorphisms was given little space and rather poorly explained — the distinction between the genetic effect of inversions in dipterans and in other organisms that have recombination in both sexes is not dealt with. And an otherwise excellent survey of genetic load is flawed by a discussion of truncation models that misses the essential point — that such models provide for a very large range of fitnesses whereas multiplicative models do not.

But these are minor caveats. Somebody else reviewing the book would doubtless find a completely different set of sins of omission (a better and more complete index, for example,

would be welcome). I read the book with great enjoyment, and can recommend it with enthusiasm. Few of us have the retentive mind of a Darwin, enabling us to push a multitude of new evolutionary facts into the front of our brains without having an equal number fall out the back. As I read, I was reminded of controversies I had quite forgotten about, and each chapter led me to think anew about the premises underlying evolutionary theories that I tend to take for granted. It will be a lot of fun to teach a course using this book. □

Christopher Wills is in the Department of Biology, University of California, San Diego, La Jolla, California 92093, USA.

Bats and their battalions

A. M. Hutson

Bats: A Community Perspective. By J. S. Findley. *Cambridge University Press: 1993. Pp. 167. £27.95, \$44.95.*

THE study of animal communities is not a new field, but has deservedly taken a higher profile in recent years. This review of the community structure of bats is particularly welcome, because the creatures comprise a diverse group, rich in fascinating aspects, even peculiarities, of distribution, population structure, morphology, feeding and other behaviour.

But the difficulties of studying bats in the field cannot be underestimated, and there is still a long way to go. Hence this review poses many more questions than it answers; bats will probably never offer the same opportunity for study that, say, birds do. Nevertheless, the author could have taken a more optimistic approach. In his preface he states that on being asked to write the book his initial reaction was that there was not much to write about. This pessimism comes across strongly in a chapter on methodology in the study of bats, in which he discusses the problems and limitations of field studies but does not highlight promising areas where there have been recent advances. This chapter is also rather sparsely referenced, a regrettable deficiency in such a key area.

In other chapters, the author provides a more investigative review of the bat assemblages found in the zoogeographic regions, the influence of resource limitation and competition, and the pattern of bat communities with reference to species richness, diversity of higher taxa, trophic and morphological diversity, and biomass and abundance. He also looks at the factors that may influence these

patterns. Inevitably, he has had to be selective in the faunas and features that he analyses and comments on. This can lead to difficulties. For example, in comparing the morphological diversity of samples from the Palaearctic and Afro-tropical regions, he resorts to fairly out-of-date species lists of Italy and Ghana. Although this choice probably makes little difference to the final analysis, it does seem to be slightly restrictive. Similarly, his comparison of the whole fauna of the European part of the former Soviet Union with the cave-hibernating fauna of the Netherlands may be misleading.

Nevertheless, this is a valuable review that indicates areas for future work. Not least among the topics for further investigation are the author's final assumptions: that in the case of forest bats, the forest's area and its refuge potential are the basic influences on diversity (species richness) and that taxonomic, morphological and trophic diversity follow in relation to species richness — there is in fact little evidence of competition or resource limitation affecting bat numbers or diversity.

Issued in the Cambridge Studies in Ecology series, this book is intended for final-year undergraduates, teachers and researchers. It will hopefully persuade them that bats are exciting biological models (enhanced by their unusual status as *K*-selected small vertebrates), offer a wide range of research opportunities and have an important place in biodiversity studies, a subject that has now achieved international recognition, if at the cost of a somewhat diffuse definition. □

A. M. Hutson is at The Bat Conservation Trust, London Ecology Centre, 45 Shelton Street, London WC2H 9HJ, UK.

Implications of the Copernican principle for our future prospects

J. Richard Gott III

Making only the assumption that you are a random intelligent observer, limits for the total longevity of our species of 0.2 million to 8 million years can be derived at the 95% confidence level. Further consideration indicates that we are unlikely to colonize the Galaxy, and that we are likely to have a higher population than the median for intelligent species.

THE Copernican revolution taught us that it was a mistake to assume, without sufficient reason, that we occupy a privileged position in the Universe. Darwin showed that, in terms of origin, we are not privileged above other species. Our position around an ordinary star in an ordinary galaxy in an ordinary super-cluster continues to look less and less special. The idea that we are not located in a special spatial location has been crucial in cosmology, leading directly to the homogeneous and isotropic Friedmann cosmological models in general relativity theory which have been remarkably successful in predicting^{1,2} the existence and spectrum of the cosmic microwave background radiation^{3,4}. In astronomy, the Copernican principle works because, of all the places for intelligent observers to be, there are by definition only a few special places and many nonspecial places, so you are likely to be in a nonspecial place. This idea can be used to estimate the likely future longevity of various observables, including that of our own species. I will discuss its implications for the far future, for the Search for Extraterrestrial Intelligence (SETI) and for space travel.

Delta t argument

Assuming that whatever we are measuring can be observed only in the interval between times t_{begin} and t_{end} , if there is nothing special about t_{now} we expect t_{now} to be located randomly in this interval. The estimate $t_{\text{future}} = (t_{\text{end}} - t_{\text{now}}) = t_{\text{past}} = (t_{\text{now}} - t_{\text{begin}})$ will overestimate t_{future} half the time and will underestimate it half the time. If $r_1 = (t_{\text{now}} - t_{\text{begin}})/(t_{\text{end}} - t_{\text{begin}})$ is a random number uniformly distributed between 0 and 1, there is a probability $P = 0.95$ that $0.025 < r_1 < 0.975$, or equivalently

$$\frac{1}{39} t_{\text{past}} < t_{\text{future}} < 39 t_{\text{past}} \quad (1) \quad (95\% \text{ confidence level})$$

Similarly,

$$\frac{1}{3} t_{\text{past}} < t_{\text{future}} < 3 t_{\text{past}} \quad (2) \quad (50\% \text{ confidence level})$$

Equation (1) tells us that the length of time something has been observable in the past is a rough measure of its robustness not only against the calamities of the past,

but also against whatever calamities may affect its observability in the future, because all that is required for equation (1) to work is that in the end *your* position as an observer turns out not to have been special. Just to illustrate, in 1969 I saw for the first time Stonehenge ($t_{\text{past}} \approx 3,868$ years) and the Berlin Wall ($t_{\text{past}} = 8$ years). Assuming that I am a random observer of the Wall, I expect to be located randomly in time between t_{begin} and t_{end} (t_{end} occurs when the Wall is destroyed or there are no longer any visitors left to observe it, whichever comes first). The Wall fell 20 years later giving $t_{\text{future}} = 2.5 t_{\text{past}}$, within the 95% confidence limits predicted by equation (1). Applying the ($P = 0.95$) delta t argument also correctly predicts that Stonehenge should still be observable today, 24 years later (as $t_{\text{future}} > 99$ years, from equation (1)). In 1977 I visited the U.S.S.R. ($t_{\text{past}} = 55$ years). Although at that time its existence into the indefinite future was generally assumed, it ended only 14 years later ($t_{\text{future}} = 0.25 t_{\text{past}}$, consistent with the limits in equation (1)). Equation (1) was satisfied not because my visit somehow caused the demise of the U.S.S.R. but simply because in hindsight we can now see that the timing of my visit was unremarkable. *Nature* has been published for 123 years; the delta t argument predicts ($P = 0.95$) its future publication will last more than 3.15 years but less than 4,800 years.

Our future longevity

Suppose, consistent with the findings of Carter⁵, intelligent species are currently being formed in the Universe at a uniform rate. By 'intelligent' species we mean one that is self-conscious and has the cognitive abilities to reason abstractly, think about the future, create art and so forth. So far, on Earth, we, *homo sapiens*, are the only species to fit this description⁶. Assume that these intelligent species are subject to some unknown extinction rate λ_0 . The distribution of ages t_p of the N_{total} intelligent species alive today is

$$N(t_p) dt_p = N_{\text{total}} \lambda_0 \exp(-\lambda_0 t_p) dt_p \quad (3)$$

as only a fraction $f = \exp(-\lambda_0 t_p)$ of those species born at an epoch t_p ago survive today. Species alive today are subject to the extinction rate λ_0 so the number of

these species becoming extinct as a function of time t_f in the future is

$$N(t_f) dt_f = N_{\text{total}} \lambda_0 \exp(-\lambda_0 t_f) dt_f \quad (4)$$

Let r_1 and r_2 be independent random numbers each distributed uniformly over the interval $[0, 1]$. For a given species alive today

$$t_p = -(\lambda_0)^{-1} \ln r_1$$

$$t_f = -(\lambda_0)^{-1} \ln r_2 \quad (5)$$

$$r_1^{(t_f/t_p)} = r_2$$

Let $Y > 0$ be a constant

$$P([t_f/t_p] > Y) = \int_0^1 r_1^Y dr_1 = 1/(Y+1) \quad (6)$$

The above probability distribution for the ratio of t_f/t_p is exactly that found earlier through what I have called the delta t argument: 50% of the time $t_f > t_p$ ($P = 0.5$ for $Y = 1$), 50% of the time $\frac{1}{3} t_p < t_f < 3 t_p$ ($P = 0.25$ for $Y = 3$ and $P = 0.75$ for $Y = \frac{1}{3}$), and 95% of the time $\frac{1}{39} t_p < t_f < 39 t_p$ ($P = 0.025$ for $Y = 39$ and $P = 0.975$ for $Y = \frac{1}{39}$); see Fig. 1.

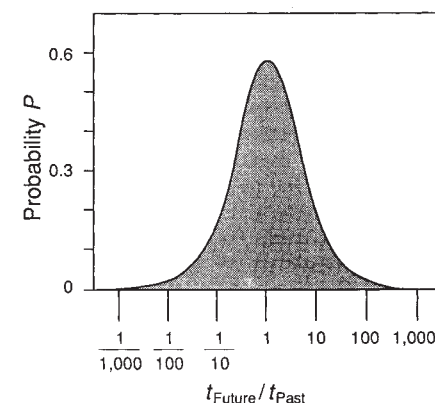


FIG. 1 Probability per unit logarithmic interval (base 10) of finding a given ratio $t_{\text{future}}/t_{\text{past}}$ for an observable from the delta t argument. The probability that $t_{\text{future}}/t_{\text{past}}$ is greater than Y is $1/(Y+1)$ [see equation (6)]. There is a 95% probability that $t_{\text{future}}/t_{\text{past}}$ lies between $(1/39)$ and 39.

Our species, *homo sapiens*, is roughly $t_p \approx 200,000$ years old. (This is conservative, as recent estimates include $>100,000$ years⁷, $>150,000$ years⁸, 200,000 years⁹ and 250,000 years⁶; if improved estimates

become available they can be substituted in an obvious way). Clearly, t_p is much less than the age of the Universe, $t_0 \approx 13 \times 10^9$ years. Using equations (1) or (6), the estimate for the future lifetime of our species is

$$5,100 \text{ years} < t_f < 7.8 \times 10^6 \text{ years} \quad (7) \\ (95\% \text{ confidence limits})$$

This gives our species a total longevity between 0.205 million and 8 million years.

The average longevity for most species is between 1 and 11 million years¹⁰⁻¹², and for mammals it is 2 million years¹³. Our direct ancestor *homo erectus* lasted only about 1.4 million years and our near relatives, the Neanderthals, flourished for roughly 200,000 years. There is thus an order-of-magnitude coincidence between the range of total longevity of our species as predicted by the delta t argument and the observed longevity of other species, particularly species like ours. Thus we should not assume that our intelligence is likely to increase our longevity vastly above that of other species.

If $\lambda_0 \ll (200,000 \text{ years})^{-1}$, only a tiny fraction $f < 200,000 \text{ years} \times \lambda_0 \ll 1$ of all intelligent observers alive at present would observe $t_p \leq 200,000$ years, and you would be unlikely to be among them. In the limit where we expect our species to live forever, λ_0 goes to 0 and $P(t_p \leq 200,000 \text{ years})$ goes to 0. What we would expect to observe in the limit as λ_0 goes to 0 is that the value of t_0 we observe goes to infinity, $t_p \approx t_0$ and $(t_0 - t_p) \ll t_0$. These would be the telltale signs that we might survive into the indefinite future. In fact we see exactly the opposite: $t_p \ll t_0$, and $(t_0 - t_p) \approx t_0$. These are the telltale signs that we can expect to last only a short time into the future $t_f \approx t_p (\times 39^{\pm 1})$.

Replace the words 'intelligent species' with intelligent genus, intelligent family, intelligent order or intelligent class, and the argument remains the same. So the estimates of t_f that I have derived apply not only to us but to any (by an argument analogous to the delta t argument there are not likely to be many) intelligent species (including intelligent machine species) descended from us.

Gould⁶ called $t_p \ll t_0$ "geology's most frightening fact", because "If humanity arose just yesterday as a small branch on a flourishing tree, then life may not in a genuine sense, exist for us or because of us. Perhaps we are only an afterthought, a kind of cosmic accident, just one bauble on the Christmas tree of evolution." I find it frightening too, but for a different reason: if $t_p \ll t_0$ then the delta t argument suggests $t_f \approx t_p (\times 39^{\pm 1})$ and thus $t_f \ll t_0$, meaning that on astronomical timescales, we and our intelligent descendants probably will not be around very long.

Population effects. When you evaluate your observations of the Universe you must take account of the selection effects

associated with the fact that you are an intelligent observer. For example, the places and times that intelligent observers are born may be limited by the laws of physics¹⁴. This is the weak anthropic principle as formulated by Carter¹⁵ and is basically just a self-consistency argument. It has had notable success in cosmology^{16,17}, giving us our best explanation of the Dirac large-numbers coincidence¹⁴. Carter⁵ has pointed out that there is a remarkable order-of-magnitude coincidence between the time required to develop intelligent life on the Earth, $t_1 = 4.5 \times 10^9$ yr measured from the formation of the Solar System, and the future main-sequence lifetime of the Sun $t_{\text{fms}} \approx 6 \times 10^9$ yr. Carter uses this fact and an ingenious argument to deduce that there must be of the order of 1 improbable event required for the formation of intelligent life. I agree; further, this coincidence is just what one would expect from the delta t argument if the intelligent life is simply formed at some random time during the main-sequence lifetime of the star. Interestingly, Carter's argument depends implicitly on the idea presented formally here: that according to the Copernican principle, among all intelligent observers (including those yet to be born) you should not be special.

Let us formalize this as the 'Copernican anthropic principle': that the location of your birth in space and time in the Universe is privileged (or special) only to the extent implied by the fact that you are an intelligent observer, that your location among intelligent observers is not special but rather picked at random. Knowing only that you are an intelligent observer, you should consider yourself picked at random from the set of all intelligent observers (past, present and future) any one of whom you could have been.

Assume therefore that you are located randomly on the chronological list of human beings. If the total number of intelligent individuals in the species is a positive integer $N_{\text{tot}} = N_{\text{past}} + 1 + N_{\text{future}}$ where N_{past} is the number of intelligent individuals in the species born before a particular intelligent observer and N_{future} is the number born after, then we expect N_{past} to be the integer part of the number $r_1 N_{\text{tot}}$ where r_1 is a random number uniformly distributed between 0 and 1. Thus:

$$(1/39) N_{\text{past}} - 1 < N_{\text{future}} < 39(N_{\text{past}} + 1) \quad (8) \\ (95\% \text{ confidence level})$$

and if $N_{\text{past}} \gg 1$

$$(1/39) N_{\text{past}} \leq N_{\text{future}} \leq 39 N_{\text{past}} \quad (9) \\ (95\% \text{ confidence level})$$

The number of human beings born so far is of the order of 70 billion (refs 18-20). By equation (9) the number of human beings yet to be born is expected to be

$$1.8 \text{ billion} < N_{\text{future}} < 2.7 \text{ trillion} \quad (10) \\ (95\% \text{ confidence level})$$

Consider the following toy model. Suppose the birth rate b is a constant and that the death rate has the constant value $d_1 < b$ before time t_{max} and the constant value $d_2 > b$ after time t_{max} . The population $p(t)$ will thus have a rate of increase $= b - d_1 = t_1^{-1}$ before time t_{max} and a rate of decrease $= d_2 - b = t_2^{-1}$ after t_{max} . The number of people born as a function of time is thus

$$N_B(t) dt = bp(t) dt \\ = bN_{\text{max}} \exp([t - t_{\text{max}}]/t_1) dt \\ \text{for } t < t_{\text{max}} \\ N_B(t) dt = bp(t) dt \\ = bN_{\text{max}} \exp([t_{\text{max}} - t]/t_2) dt \\ \text{for } t > t_{\text{max}} \quad (11)$$

Regardless of the values of t_1 and t_2 , 50% of observers are born at times when the population $p(t)$ is within a factor of 2 of its maximum value of N_{max} (and 95% of observers are born when the population is within a factor of 20 of N_{max}). It is thus interesting that the current population of the Earth is over 5 billion and many projections of future growth show the population topping out at ~8 to 12 billion²¹⁻²³. Estimates of the theoretical maximum population for the Earth include 20 billion²¹ and 40 billion²⁴. Exponential growth and decline is common in biological systems, so you should not be surprised to be born in an epoch with problems of overpopulation. The total number of people born before t_{max} is $N_1 = bN_{\text{max}}t_1$ and the number of people born after t_{max} is $N_2 = bN_{\text{max}}t_2$, so $N_1/N_2 = t_1/t_2$. In the limit where t_2 goes to infinity, this model crudely follows a logistic curve, a period of exponential growth followed by a long period of equilibrium where the birth and death rates are equal. But this is not allowed because if you find yourself on the rising exponential you are a member of the set N_1 , and we require that $N_2 < 39N_1$ in order that your good luck should not be more spectacular than 2.5%; this means that $t_2 < 39t_1$, and as you are likely to be born near t_{max} , that $t_f \approx 39t_p$ (just as in the delta t argument). But the set N_2 to which you do not belong can be as small as 0, which means that the only lower limit on t_2 is 0. In the limit $t_2 = 0$ (sudden extinction) you are likely ($P = 0.95$) to be born between 3.7 and 0.025 e-folding times (t_1) of the end, giving a lower limit of $t_f > 0.025t_1 \approx t_p/40 \ln(N_{\text{max}})$.

We might reasonably expect, as a first guess, that an eventual population crash would roughly mirror its rise but perhaps be multiplied by a scale factor in time. In this case we would still expect the 95% confidence level upper limit on t_f to be $\sim 39t_p$ just as in the delta t argument (because if the fall mirrored the rise but was stretched out by a factor of 39 in time, then 39/40 of the people would be born

on the declining portion of the curve). The 95%-confidence-level lower limit on t_f can be calculated directly by noting that the 95%-confidence-level lower limit on the number of future human births is 1.8 billion (equation (10)). At current rates²⁵ it will take only about 12 years for another 1.8 billion people to be born (assuming births continue without abatement before a sudden extinction). This extremely pessimistic lower limit would need a double dose of bad luck, bad luck at the 2.5% level coupled with an instantaneous extinction. The number of years is so short because a reasonable fraction, 7.7%, of all the people who have ever lived are alive today^{18-20,25}. This is a dangerous situation. Thus, including population effects,

$$12 \text{ years} < t_f < 7.8 \text{ million years} \quad (12) \\ (95\% \text{ confidence level})$$

Disturbingly, even extraordinarily low values of t_f cannot be confidently excluded ($P=0.95$) but high values of t_f , such as many billion years, which we might hope for, can be.

Ehrlich and Ehrlich²⁴ propose three possible future population scenarios for the human race: (1) topping out at 10 billion in the next century and then dying out; (2) topping out at 10 billion in the next century and then collapsing to a few hundred thousand people eking out a livelihood on an impoverished planet for the next 4 million years; and (3) a sustainable stable population of 1 billion for the next 4 million years. The number of future births implied by these scenarios are roughly (1) 10 billion, (2) 30 billion and (3) 40×10^{12} . Unfortunately, only the two pessimistic cases (1) and (2) are within the limits in equation (10) (see Fig. 2). Combining $N_{\text{future}} < 2.7 \times 10^{12}$ (equation (10)) with the current rate of 145 million births per year²⁵ we find $t_f < 19,000$ years unless the rate of births drops. If we wish to stretch our survival out to the upper limit of 7.8 million years (from equation (12)) we require the average rate of births to drop by a factor of more than 400. Although an optimist might hope for this to be produced by extraordinary means such as genetic engineering producing a greatly increased human lifetime, a pessimist would note that any civilization potent enough to produce a large linear increase in its life expectancy would be potent enough to produce an exponential increase in its numbers as well, and argue that such a drop could be more easily produced simply by a population crash.

Equation (9) claims that it is likely that you are not in either the first 2.5% or the last 2.5% of the chronological list of human beings. Can you avoid this conclusion by arguing that you occupy a special position on the list because you are born into an epoch where the level of sophistication is great enough to know equation (9)? Well, if you are now over

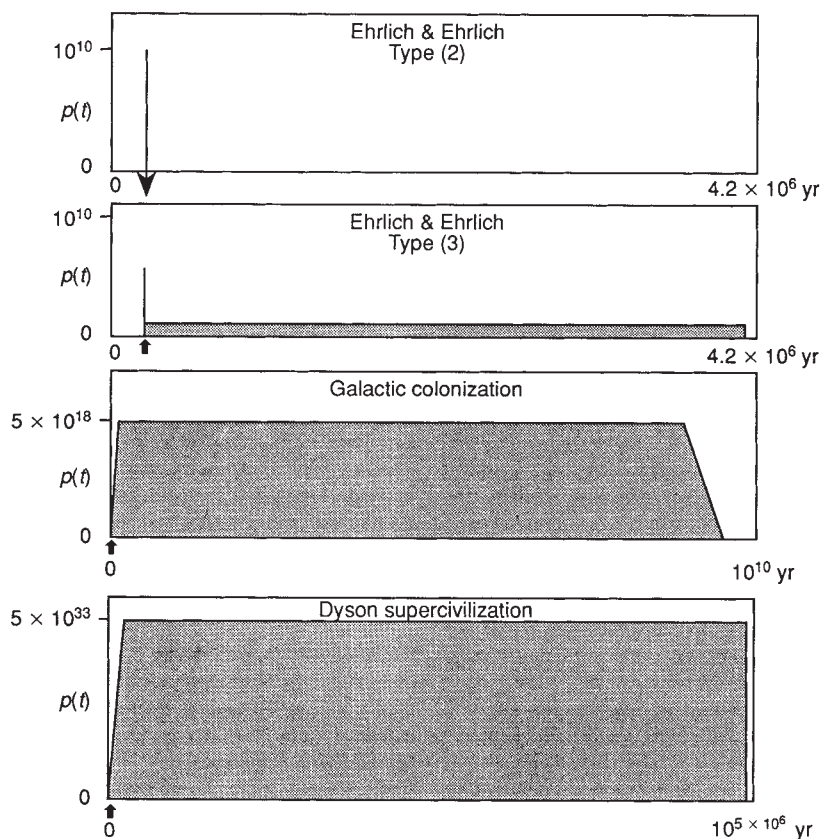


FIG. 2 Population against time for various scenarios discussed in the text shown on linear scales. In the pessimistic Ehrlich & Ehrlich Type (2) scenario, the population reaches 10 billion in the next century and then collapses to a few hundred thousand for the next 4 million years; on this scale, only the population spike near the present is visible. In all but the Type (2) scenario your position (black arrow) turns out to be extraordinarily lucky to be in the first tiny fraction of the intelligent observers in our lineage.

12 years old, more than 1.8 billion people have already been born after you, so you already know that you are not in the last 2.5% of the chronological list. In another 12 years, with the current rate of births, everyone alive today will be off the last 2.5% of the chronological list. So bad luck is required for you to be on the last 2.5% of the list. If you are an optimist and believe that civilization will go onward and upward from here, then any future humans should live in a civilization advanced enough to remember or rederive equation (9); so of all those observers who live in epochs sophisticated enough to apply equation (9), less than 2.5% of them can be in the first 2.5% of the entire chronological list (because they occupy all of the chronological list except for a segment at the beginning).

In such an optimistic scenario if $N_{\text{future}} > 2.7 \times 10^{12}$, you would have to ask yourself why, of all the more than 2.7×10^{12} humans who will live in epochs where equation (9) or its variants are known, you are so lucky ($P < 0.002$) as to be among the 5.4 billion people alive when it is discovered. For example, you live in an epoch

where Copernicus's theory is known, but you were not lucky enough to have been around when it was discovered, and in such an optimistic scheme it would be likely for you to be in the same position with respect to equation (9). The only hope to force you into the first 2.5% of the chronological list is to live in a type (2) scenario where civilization collapses in the near future and we return just as before to a hunter-gatherer phase (which forgets the equation and is not sophisticated enough to rederive it) which one might hope would last a long time. But this will not work either, because without civilization we would lose our possibility of being especially protected against extinction and we should at that point have an expected future longevity of about 2 million years or less, like other hominid species. The population in the final hunter-gatherer phase should be low so that future births should be of order 30 billion (consistent with equation (9)). (In such a situation, the civilization, by Carter's⁵ argument, should form at some random time during the lifetime of the species and the delta t argument should apply). Being in

the first 2.5% of the chronological list requires at least good luck at the 2.5% level in all these cases.

Any biases due to the fact that we live in a technological civilization we expect to be small in any case, because populations are so high during technological civilizations that we expect a substantial fraction of all intelligent observers to be born into them.

Prospects for the far future

The probability that we will colonize the Galaxy (increasing our population by a factor of at least 10^9) is of order $P \leq 10^{-9}$, as in such a situation we expect $N_{\text{future}} > 10^9 N_{\text{past}}$ and $P(N_{\text{future}} > 10^9 N_{\text{past}}) \leq 10^{-9}$. (Seeding other worlds with microorganisms²⁶ might be possible, but by Carter's⁵ argument only a small fraction would ever develop intelligent life. Even if one entertained the notion that life on Earth was seeded in this way²⁶⁻²⁸, the typical delay of several billion years to evolve intelligent life when it does occur guarantees that the number of direct 'ancestor' civilizations in our lineage must be at most a few, and, using an argument similar to the delta t argument, the number of our 'descendant' intelligent species is also not likely to be large). The probability that we will build a Dyson²⁹ sphere of O'Neill³⁰ solar-powered space colonies surrounding the Sun (using all the Sun's radiant energy and multiplying our population by a factor of at least 10^8) is of order $P \leq 10^{-8}$. The probability of our establishing a Kardashev type III civilization, defined as one that uses the energy output of its entire galaxy by placing a Dyson sphere around each star (multiplying our population by a factor of at least 10^{17}) is of order $P \leq 10^{-17}$.

Dyson³¹ showed that operating at lower and lower temperatures as the Universe expands (thinking ever more slowly, and interspersing periods of hibernation), intelligent life could in principle continue forever, having an infinite number of thoughts on only a finite amount of energy. As an example, consider Linde's³² chaotic inflationary³³ cosmology where our Universe may continue its normal expansion for $\sim 10^{5 \times 10^6}$ years. The amount of effective conscious time elapsed would be $10^{1.25 \times 10^6}$ years because of the slow thinking and hibernation. Because the mass within the horizon grows linearly with time in the Linde model up to $10^{5 \times 10^6}$ years, even with an extremely pessimistic probability for the formation of intelligent life on a habitable planet (say $10^{-4,000}$) there would still be of the order of $10^{5 \times 10^6}$ intelligent civilizations in our entire inflationary domain that would eventually become visible. Intelligent observers with life-expectancies longer than 10^2 conscious years must not be sufficiently common to dominate the total, otherwise you would be likely to be one. Elimination of all current causes of death other than murder and suicide

would only increase life-expectancies to 10^4 years and even extraordinary increases in lifespans (of up to say 10^{100} conscious years) would still require the Dyson supercivilization to have over time of order $10^{1.25 \times 10^6}$ individuals and our probability of becoming a Dyson supercivilization would be less than of order $10^{-1.25 \times 10^6}$; still there could be as many as $\sim 10^{3.75 \times 10^6}$ Dyson supercivilizations in the Universe. So some intelligent life may last into the far future; it is just not likely to be us or our descendants.

In the limit where (cosmology permitting) a supercivilization is able to accumulate an infinite amount of elapsed conscious time and an infinite number of intelligent observers (N_{tot}), the fraction of ordinary civilizations such as ours that will develop into such a supercivilization must go to zero so that the set of observers born on the original home planet is not an infinitesimal minority of all intelligent observers.

Implications for SETI

Colonization is not important in the sense that galactic colonists and their descendants must not dominate the numbers of intelligent observers in the Universe (otherwise you would be likely to be one). Significantly³⁴, this explains why we should not be surprised that we have not been colonized by extraterrestrials. Assume 10^9 habitable planets in the Galaxy (surely optimistic). Carter's⁵ argument shows that the fraction of these that will develop intelligent life is at least an order of magnitude and perhaps many orders of magnitude less than 1. As the main-sequence lifetime of their star t_{ms} is of the order of 10^{10} years, the rate at which intelligent civilizations are forming in the Galaxy is $\eta < 0.01 \text{ yr}^{-1}$. If the average longevity of these civilizations for radio-transmission is $\langle L \rangle$ then (as colonization is not important) by the Drake equation^{35,36} we expect to be able to observe within our Galaxy $N_0 = \eta \langle L \rangle < 0.01 \text{ yr}^{-1} \langle L \rangle$ civilizations transmitting now. You are born into a radio-transmitting civilization, so you should be picked at random from the set of observers in radio-transmitting civilizations. Assume our radio transmission longevity is L_j (radio transmission may end because we become extinct, technological civilization comes to an end, or we simply move on to a different method of communication). Then by the delta t argument we know that $L_p = r_1 L_j$ where $L_p = 105$ years is the past longevity of radio transmission on Earth, and r_1 is a random number uniformly distributed between 0 and 1. Arrange all N radio-transmitting civilizations in a catalogue in order of their radio longevity so that for all i , $L_i \leq L_{i+1}$. Assume that the number of intelligent observers in a radio-transmitting civilization is proportional to L (equivalent to

assuming a civilization's rate of births is not correlated with L ; if it were positively correlated, which might seem natural, then the limits would be even stronger). As you are randomly located on the list of observers in the catalogue

$$\sum_{i=1}^j L_i = r_2 \sum_{i=1}^N L_i = r_2 \langle L \rangle N$$

$$\leq j L_j \leq N L_j = N L_p / r_1 \quad (13)$$

where r_2 is a random number uniformly distributed between 0 and 1. Thus

$$\langle L \rangle \leq L_p / (r_1 r_2) \quad (14)$$

The probability $P = 0.95$ that $(r_1 r_2) > 0.0087$ so

$$\langle L \rangle < 12,100 \text{ years}, \quad N_0 < 121 \quad (95\% \text{ confidence level}) \quad (15)$$

independent of the form of the distribution of the L s, and considerably lower than Cameron's³⁷ estimate of $\langle L \rangle = 10^6$ years.

Thus there is some possibility that a radio search for other civilizations in our Galaxy or others could be successful, but a targeted radio search of 1,000 nearby stars is not likely to succeed. The upper limit on N_0 is generous because Carter's⁵ argument implies that the probability of forming an intelligent species on a habitable planet around a given star during its main-sequence lifetime may be many orders of magnitude below unity rather than the optimistic one order of magnitude below unity that we adopted.

As a human being today the probability is large ($P = 0.97$) that you were born in a country with a population of more than the median value of 6.3 million²⁵. For the same reason, if there is a large spread in the populations of intelligent species, which we would expect for noninteracting opportunistic species, then it is likely that you, as a random intelligent observer, would find yourself in an intelligent species with a population larger than the median. (In the canonical $a = 0.2$ lognormal distribution which best fits a wide variety of observed species-abundance curves, 98.6% of all individuals come from species having populations above the median value)³⁸⁻⁴². Civilizations significantly larger than our own must be sufficiently rare that their individuals do not dominate the total. Thus, we do not expect to see a Dyson sphere civilization within our Galaxy, or a Kardashev type III civilization within the current observable horizon.

Implications for space travel

Why is our probability of colonizing the Galaxy so low given that it would be very good for our survival prospects to expand our habitat vastly and that we already know how to travel in space? Although we have been around for 200,000 years or so, civilization (with cities and writing) has only been around for 5,500 years and

technological civilization sophisticated enough to engage in space travel for only 32 years. But the delta t argument tells us that the capacity and motivation to engage in space travel may be with us for only of the order of another 32 years ($\times 39^{\pm 1}$). The Cold War which was responsible for the space race in the first place is now over. Human flights to the Moon lasted for only four years, and human activity in space has unfortunately retreated at present to low Earth orbit. The delta t argument suggests that there may be only a brief window of opportunity for space travel during which we will in principle have the capability to establish colonies (which could in turn establish further colonies). If we let that opportunity pass without taking advantage of it we will be doomed to remain on the Earth where we will eventually go extinct. So far, not a single human being has been born outside the gravitational potential well of the Earth.

The methods that I have used here are very conservative; if the results are dramatic it is only because the facts are dramatic ($t_p \ll t_0$). This paper only points out and defends the hypothesis that you are a random intelligent observer. Sometimes we say that the future is unpredictable. Another way to look at this is to say that at birth you have no information on where fractionally in the chronological list of human beings you will in the end turn out to be. You are no more likely eventually to turn out to be in the first 2.5% of the chronological list of human beings than you are to be in the first 2.5% of the alphabetical list of human beings in your home town's telephone book. Short of having actual data on the longevities of other intelligent species, this hypothesis is argu-

ably the best we can make. At present we have no data sufficient to reject this hypothesis and it does explain a number of facts: that you were born during a period of high population and that the Earth has not been previously colonized by extraterrestrials. Like any good scientific hypothesis, this hypothesis is falsifiable, either immediately by discovery of intelligent radio signals from a civilization around a nearby star; or eventually, if more than 2.7×10^{12} more human beings are born. If you believe that our intelligent descendants will last 10 billion years and colonize the Galaxy, you must believe that you will, in the end, turn out to have been very lucky to have been in the first tiny fraction of the members of our intelligent lineage (see Fig. 2). If you were not lucky enough to find yourself on the first page of the phone book or were not even born on January 1, can you feel comfortable assuming that you will turn out to be even luckier in the ultimate chronological list? You should be suspicious of any claim that future events will conspire to make you *in the end* turn out to be exceptionally lucky, like the claim that you will win the lottery tomorrow or get rich by participating in a chain letter you have received.

What about the future of all life on Earth? Darwin⁴³ said "And of the species now living very few will transmit progeny of any kind to a far distant futurity. . . . As all the living forms of life are the lineal descendants of those which lived long before the Silurian epoch, we may feel certain that the ordinary succession of generations has never once been broken, and that no cataclysm has desolated the whole world. Hence we may look forward with some confidence to a secure future

of *equally* inappreciable length" [italics mine]. This is essentially the delta t argument applied to our position among all living things on earth. If our species does become extinct sometime within the next few million years, and we do not colonize space and are not potent enough to destroy all life on Earth either, then we will indeed be like other species (albeit especially interesting) and we might indeed be expected to occupy a random position in the history of life on earth. Life on Earth began over 3.6 billion years ago, and we might expect it to last another 6 billion years until the Sun becomes a red giant, in agreement with equation (1) and Darwin's prediction.

The odds are against our colonizing the Galaxy and surviving to the far future, not because these things are intrinsically beyond our capabilities, but because living things usually do not live up to their maximum potential. Intelligence is a capability which gives us in principle a vast potential if we could only use it to its maximum capacity, but so does the ability to lay 30 million eggs as the ocean sunfish does⁴⁴. We should know that to succeed the way we would like, we will have to do something truly remarkable (such as colonizing space), something which most intelligent species do not do.

After completing this paper, I have learned that ideas on future human population similar to some of those presented here were discussed by Brandon Carter in a 1983 talk, although never in print (see treatment by Leslie⁴⁵⁻⁴⁷), as well as independently proposed by Nielson⁴⁸. □

J. Richard Gott III is at the Department of Astrophysical Sciences, Princeton University, Princeton, New Jersey 08544, USA

- Gamow, G. *Phys. Rev.* **74**, 505-506 (1948).
- Alpher, R. A. & Herman, R. C. *Nature* **162**, 774-775 (1948).
- Penzias, A. A. & Wilson, R. W. *Astrophys. J.* **142**, 419-421 (1965).
- Mather, J. C. et al. *Astrophys. J.* **354**, L37-L40 (1990).
- Carter, B. *Phil. Trans. R. Soc. A* **310**, 347-363 (1983).
- Gould, S. J. *Wonderful Life*, 44-320 (Norton, New York, 1989).
- Caroll, R. *Vertebrate Paleontology and Evolution*, 475-476 (Freeman, New York, 1988).
- Stringer, C. B. *Scient. Am.* **263**, 98-104 (1990).
- Cann, R. L., Stoneking, M. & Wilson, A. C. *Nature* **325**, 31-36 (1987).
- Wilson, E. O., *Biodiversity* (ed. Wilson, E. O.) 8 (National Academy Press, Washington DC, 1988).
- Raup, D. M. *Biodiversity* (ed. Wilson, E. O.) 54 (National Academy Press, Washington DC, 1988).
- Raup, D. M. *Science* **231**, 1528-1533 (1986).
- Stanley, S. M. *Proc. natn. Acad. Sci.* **72**, 646-650 (1975).
- Dicke, R. H. *Nature* **192**, 440-441 (1961).
- Carter, B. *Confrontation of Cosmological Theories with Observation* (ed. Longair, M. S.) 291-294 (Reidel, Dordrecht, 1974).
- Carr, B. J. & Rees, M. J. *Nature* **278**, 605-612 (1979).
- Barrow, J. D. & Tipler, F. J. *The Anthropic Cosmological Principle*, 231-248 (Clarendon, Oxford, 1986).
- Desmond, A. *Population Studies: Selected Essays and Research* (ed. Kammeyer, K. C. W.), 436-450 (Rand McNally, Chicago, 1969).
- Biraben, J.-N. *Population 34e Année*, no. 1, 13-25 (1979).
- Thatcher, A. R. *Guinness Book of World Records 1991* (ed. McFarland, D.) 202 (Facts On File, New York, 1990).
- McEvedy, C. & Jones, R. *Atlas of World Population History*, 351 (Penguin, Middlesex, 1978).
- Demeny, P. *World Population and U.S. Policy* (ed. Menken, J.) 65 (Norton, New York, 1986).
- United Nations Population Projections: Problems and Solutions, Workshop on Population Projections, Budapest, 1980, 118 (United Nations, New York, 1981).
- Ehrlich, P. R. & Ehrlich, A. H. *The Population Explosion* 16-237 (Simon and Schuster, New York, 1990).
- Haub, C., Kent, M. M. & Yanagishita, M. 1991 *World Population Data Sheet* (Population Reference Bureau, Washington DC, 1991).
- Crick, F. H. C. & Orgel, L. E. *Icarus* **19**, 341 (1973).
- Arrhenius, S. *Die Umschau* **7**, 481 (1903).
- Gold, T. *Air Force and Space Digest*, May, 65 (1960).
- Dyson, F. J. *Science* **131**, 1667-1668 (1960).
- O'Neill, G. K. *Physics Today* **27**, 32-40 (Sept. 1974).
- Dyson, F. J. *Rev. mod. Phys.* **51**, 447-460 (1979).
- Linde, A. D. *Inflation and Quantum Cosmology*, 139-141 (Academic, Boston, 1990).
- Guth, A. H. *Phys. Rev. D* **23**, 347-356 (1981).
- Hart, M. H. & Zuckerman, B. *Extraterrestrials—Where Are They?* (Pergamon, New York, 1982).
- Drake, F. & Sobel, D. *Is Anyone Out There?* 52-68 (Delacorte, New York, 1992).
- Shklovskii, I. S. & Sagan, C. *Intelligent Life in the Universe*, 409-418 (Dell, New York, 1966).
- Cameron, A. G. W. *Sky and Telescope* **26**, 258-261 (1963).
- May, R. M. *Ecology and Evolution of Communities* (eds Cody, M. L. & Diamond, J. M.) 81-120 (Harvard Univ. Press, Cambridge, MA, 1963).
- Preston, F. W. *Ecology* **29**, 254-283 (1948).
- Preston, F. W. *Ecology* **43**, 185-215 (1962).
- Preston, F. W. *Ecology* **43**, 410-432 (1962).
- Whittaker, R. H. *Taxon* **21**, 213-251 (1972).
- Darwin, C. *On the Origin of Species*, 1859 (reprinted by Harvard Univ. Press, Cambridge, MA, 1964).
- McFarland, D. *Guinness Book of World Records 1991*, 42 (Facts On File, New York, 1990).
- Leslie, J. *Bull. Canad. nucl. Society*, **10** (3), 10-15 (1989).
- Leslie, J. *The Philosophical Quarterly*, **40**, 65-72 (1990).
- Leslie, J. *Mind* **101.403** 521-540 (1992).
- Nielsen, H. B. *Acta phys. Polon.* **B20**, 427-468 (1989).

Visualization of coherent nuclear motion in a membrane protein by femtosecond spectroscopy

Marten H. Vos*, Fabrice Rappaport*, Jean-Christophe Lambry*, Jacques Breton† & Jean-Louis Martin*‡

* Laboratoire d'Optique Appliquée, INSERM U275, Ecole Polytechnique ENSTA, 91120 Palaiseau, France

† Service de Biophysique, Département de Biologie, CEN Saclay, 91191 Gif-sur-Yvette Cedex, France

Multicolour near-infrared femtosecond experiments of a genetically modified bacterial reaction centre show that the phase of two excited-state vibrational modes is conserved for a period of picoseconds over a large range of temperatures. The direct visualization of low-frequency nuclear vibrations in this protein, which is embedded in its natural membrane, implicates coherent nuclear motion in the primary electron transfer reaction in functional reaction centres.

THE specific function of protein macromolecules relies on their internal nuclear dynamics which allow them to adopt conformations differing in structure and properties. Identification of these functionally important motions is a major goal in biological chemistry. Such motions take place over a long time period. The fastest dynamics occur in the subpicosecond or picosecond timescale. In the latter case, the main insight comes from molecular dynamics simulations^{1–5}, which predict a key role for low-frequency ($1\text{--}100\text{ cm}^{-1}$, $0.05\text{--}5 \times 10^{12}\text{ Hz}$) global protein motions. An obvious example of the mechanistic importance of nuclear motions is the binding of small ligands to iron in haem proteins. Here, both thermal fluctuations and relaxations of the protein moiety are thought to govern the binding rate. This reaction involves nuclear motion over a low-energy barrier. This situation may be quite ubiquitous in biological systems because most of the catalytic reactions occur in the low-barrier environment. Other examples of such situations are the isomerization reactions that initiate the process of vision or trigger a cascade of molecular events leading to the transfer of H^+ ions across the membrane in bacteriorhodopsin. As expected for low-barrier reactions, the above processes can be very rapid. Under certain circumstances, the time required to move from the reactants potential energy surface to the product surface may approach the timescale of low-frequency nuclear motions. If such motions are along the reaction coordinate, the reaction approaches the adiabatic limit.

Perhaps the most striking example of a fast biological reaction is the primary step in electron transfer within reaction centres from purple bacteria. The primary charge separation is an example of a physiological reaction occurring on a timescale similar to the vibrational period of low-frequency protein motion. This electron transfer reaction occurs in $\sim 3\text{ ps}$ at 300 K (refs 6, 7) and 2–4 times faster at low temperature⁸. For such a fast reaction, the conventional hypothesis on which most theoretical treatments of electron transfer have been based, that is the temporal separation between electronic reorganization and vibrational relaxation, is at least questionable. This point remains an open question and has been discussed in detail^{9–11}. The most popular hypothesis in the standard picture of electron transfer in the condensed phase has been to assume that nuclear motion along the reaction coordinate suffers many collisions, that is, changes in velocity and energy, before the reaction occurs. Following this view, such friction forces efficiently destroy the phase of the elementary vibrational motions, leading

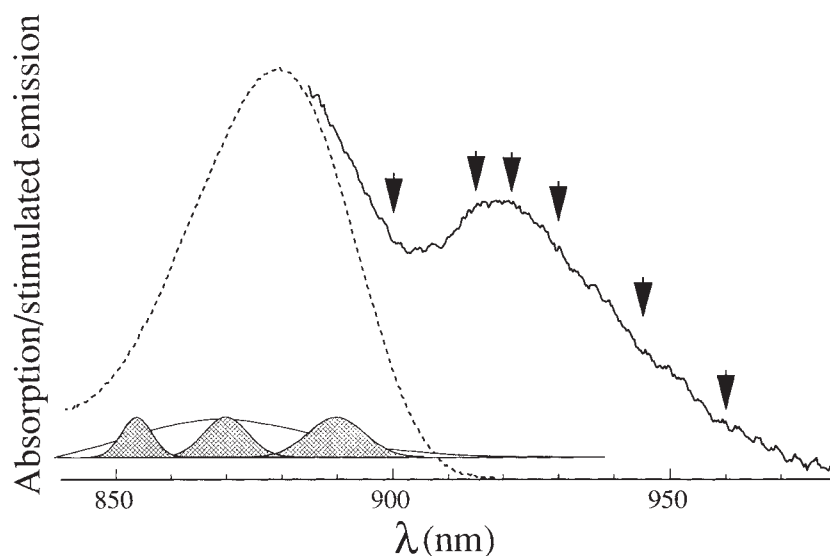
to a complete loss of phase coherence on a timescale that is short compared to the electron transfer reaction.

If this strong assumption does not hold, that is if the dephasing rates are comparable to the timescale of electron transfer, the standard classic or semiclassical description will no longer be valid. Here we demonstrate the presence of phase coherence of low-frequency motions on the timescale of electron transfer. We report the direct experimental visualization of low-frequency vibrations in a protein embedded in its natural membrane. We use a (modified) reaction centre from the purple bacterium *Rhodobacter capsulatus*, where the excited state of the primary donor can be physiologically populated and where vibrations can be accurately synchronized by a femtosecond light pulse. The time evolution of the stimulated emission of the primary donor shows oscillatory modulation on a picosecond timescale. We demonstrate the vibrational origin of these oscillations by using multicolour near-infrared femtosecond spectroscopy. Furthermore, the dependence on both the excitation and probe wavelengths of the frequency, phase and amplitude of the oscillation reveals that they reflect vibrational motion on the excited state rather than the ground state and low-frequency modes (15 and 77 cm^{-1}), rather than beating between high-frequency modes. Whereas the oscillatory features are most prominent at cryogenic temperatures, they can also be observed at higher temperatures. The ability to observe the protein vibrations is a consequence of their phase conservation for a few picoseconds.

Most processes in proteins are governed by motions that are considered as stochastic, that is devoid of phase memory. But the phase memory of vibrational motion is lost by quasi-elastic (vibrational dephasing) and inelastic (vibrational relaxation) collisions. Even in systems as condensed and complex as proteins, the dephasing processes take as long as picoseconds^{1,4,12}. As the vibrational energy density is mostly contained in the frequency range below 100 cm^{-1} (refs 1–3), we may expect to observe coherent-like wave packet motions in proteins on a picosecond timescale in a way similar to those in simple alkali molecules^{13–15}. The picture of wave packet motion has already been used to describe the retinal isomerization reaction in bacteriorhodopsin¹⁶, but in this case critical damping prevented the observations of low-frequency oscillations¹⁷. We have previously reported oscillatory features in bacterial reaction centres that persisted, at cryogenic temperatures, for several picoseconds after excitation with a femtosecond pulse¹⁸. But we did not prove that they reflected vibrational nuclear motion. Here we have been able to discriminate between several mechanisms that

‡ To whom correspondence should be addressed.

FIG. 1 Low-temperature (10K) optical properties of the P band of reaction centres of the D_{LL} mutant of *R. capsulatus*. The reaction centre of wild-type photosynthetic bacteria is a membrane-bound protein. Six pigments are bound in a near C_2 symmetry arrangement to two of its subunits (L and M)^{31,32}. Population of the lowest excited singlet state of the complex, P^* , results in a transmembrane electron transfer from the dimeric bacteriochlorophyll (Bchl) donor P to the bacteriopheophytin acceptor H_L in ~ 3 ps at room temperature and ~ 1 ps at 10K along the L branch only^{6-8,28,33}. The charge pair is stabilized by subsequent slower reactions. In the D_{LL} mutant, the D α -helix in the M subunit has been genetically symmetrized with respect to its L analogue, which contains 13 differing amino acids. This resulted in the removal of H_L ³⁴. Any electron transport is thus inhibited and P^* lives for several hundred picoseconds before decaying to the ground state¹⁹. In the bacterial mutant the β and α structural genes coding for light-harvesting (LH) complex II have been deleted from the chromosome of the *R. capsulatus* bacterial strain³⁵. Site-directed mutations near the putative Bchl-binding site in LHI have resulted in the loss of this complex from the membranes mainly due to post-transcriptional processes³⁶. Absorption spectra of the mutant preparations confirm the absence of LHI and LHI complexes³⁶. The D_{LL} mutant has been constructed as described above. Chromatophore membranes were prepared by disrupting cells in a French pressure cell and subsequent purification by sucrose density gradient centrifugation. The ground-state absorption spectrum was recorded in a polyacrylamide gel³⁷. For the femtosecond experiments the membranes were suspended in a 50/50 (v/v) mixture of glycerol and buffer (20 mM Tris pH 8.0). Stimulated emission, recorded 10 ps after excitation, (solid line) is displayed as the negative of the measured absorption change to aid comparison with ground-state



absorption (dashed line). The high-energy side of the stimulated emission band, at wavelengths shorter than 910 nm, overlaps with the ground-state transient bleaching. The data of Figs 1, 2 and 6 have been obtained in femtosecond pump-probe experiments¹⁸ using an 80 fs Fourier transform-limited pump pulse at 870 nm and a 30 fs continuum probe pulse. Arrows indicate the probe wavelengths at which the kinetic data were recorded. The hatched bands indicate the spectral profiles of the pump pulses used in the experiments of Fig. 4; the dotted band those of Fig. 5b,c. All experiments were done in a convection cryostat. With the exception of the experiment of Fig. 6, the temperature was maintained at 10K.

might give rise to oscillatory features in optical transients. Two principal mechanisms are envisaged: (1) electronic quantum beats, that is, oscillation of population density between closely-lying electronic states; and (2) vibrational coherence, that is, in-phase nuclear motion, which may directly modulate the spectrum and give rise to a time-dependent transition probability to another state. If the latter applies, vibrational relaxation must take place on a timescale of a few picoseconds, whereas in the former mechanism the intramolecular vibrational energy redistribution can be very fast. In the case of vibrational coherence, impulsive perturbation may (de)populate vibrational levels in both the excited state and the ground state and any observed frequency may correspond to the fundamental frequency of an

activated mode, to second or even higher harmonics of the fundamental frequency, or to beatings between higher frequency modes.

We studied the spectral properties of the oscillating features in the near infrared-stimulated emission band of reaction centres of the D_{LL} mutant of *R. capsulatus*. These experimental conditions are chosen for convenience of interpretation: in bacterial reaction centres the stimulated emission band displays almost no spectral overlap with the ground state absorption, and in the D_{LL} mutant no reactions occur on the timescale of a few picoseconds¹⁹. Furthermore, the reaction centre proteins used are maintained in their natural membrane²⁰. As the membrane provides a molecular environment with reduced degrees of free-

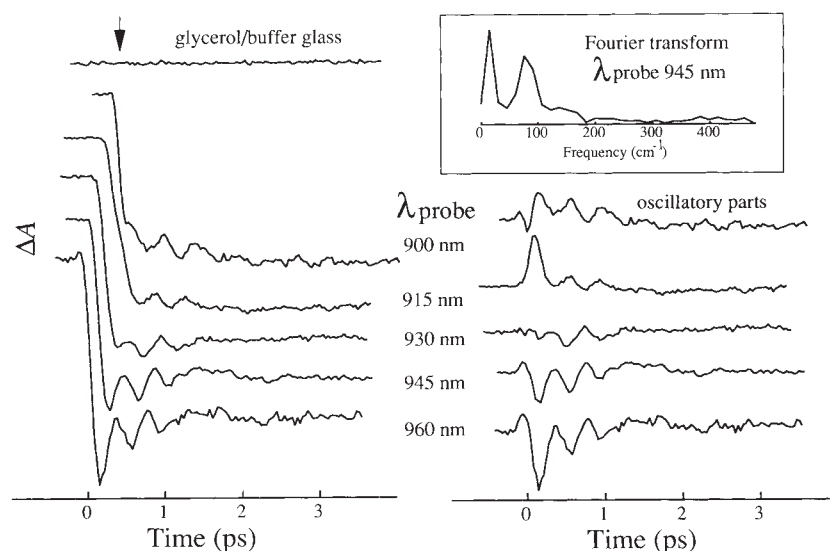


FIG. 2 Dependence of the oscillations in the stimulated emission on the probe wavelength. The traces are normalized to the average amplitude of the overall signal. The oscillatory part at each probe wavelength is obtained by taking the residuals of a fit to a step function convoluted with the instrument response function. Discrete Fourier transformation describes the input signal $I(t)$ by its amplitudes A and phases ϕ as a function of the frequency ν as $\sum_{\nu} \text{Re}(A_{\nu} e^{i(2\pi\nu t - \phi_{\nu})})$ (Re : real part) and was taken, for all probe wavelengths, over a 2.13-ps data interval (64 points), yielding a spectral resolution of 15 cm^{-1} . The Fourier transform (FT) window was started at $t=0.10$ ps, excluding the pump-probe overlap time interval. The 80 fs pump pulse duration determines the vibrational bandwidth of the experiment at $\sim 70 \text{ cm}^{-1}$. The spectral region above $\sim 200 \text{ cm}^{-1}$ represents the noise level of the data. The FT amplitudes of the oscillatory parts all peak at 15 cm^{-1} and 77 cm^{-1} ; the inset shows the FT amplitude for $\lambda \text{ probe} = 945 \text{ nm}$. Upper left: control experiment at 10K ($\lambda \text{ probe} = 930 \text{ nm}$) using the same cuvette containing only the buffer/glycerol mixture. The arrow indicates $t=0$. The trace is plotted on the same amplitude scale as the protein sample (930 nm trace).

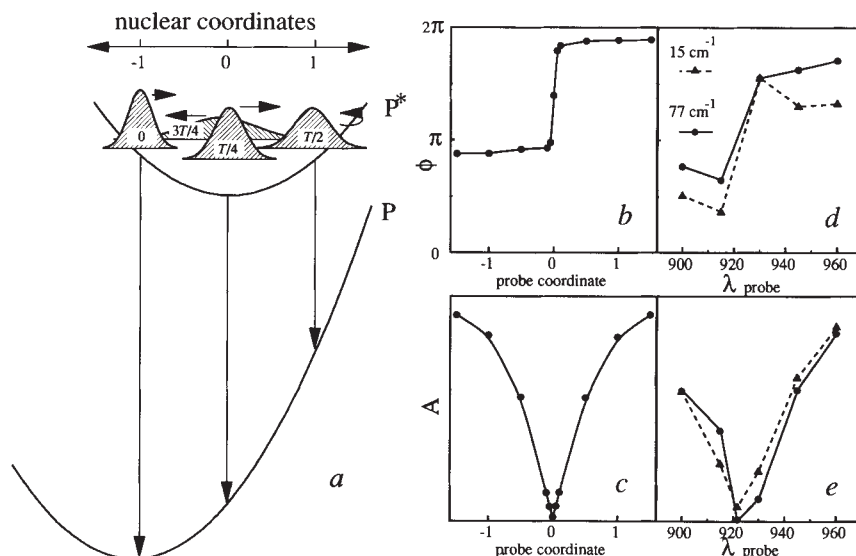
FIG. 3 One-dimensional scheme of wave packet motion on the potential energy surface of the excited state P^* on excitation near the turning point (a). The initial width of the wave packet is determined by the spectrum of the excitation pulse. While propagating over the potential energy surface, the wave packet broadens because of vibrational dephasing and relaxation and possibly anharmonicity. At each turning point the packet passes once per period ($t=0, T, \dots$, and $t=T/2, 3T/2, \dots$, respectively; T is the fundamental period), giving rise to components of the well frequency with opposite phases³⁸. At the bottom of the well, the packet passes twice per period ($t=T/4, 3T/4, 5T/4, \dots$), giving rise to a component at the double frequency of the well. In functioning reaction centres, the population of P^* diminishes by crossing to the P^+ surface (not drawn), but this reaction is blocked in the D_{LL} mutant. Only one of the many degrees of freedom is depicted; our experiments demonstrate coherent motion in at least two dimensions. The motion is probed by stimulated emission to the ground state. Owing to the displacement of the surfaces,

a wavelength dependence is predicted. This was simulated for a harmonic oscillator of 75 cm^{-1} , initially populated with a 130 cm^{-1} (10 nm at $\lambda = 870\text{ nm}$) broad wave packet centred at a nuclear coordinate defined as -1 , at an excess energy of 500 cm^{-1} . Damping was modelled by diffusion broadening of the wave packet. The wave packet density at different nuclear coordinates was calculated as a function of time delay. The stimulated emission was simulated by integration over a time window corresponding to the instrument response function and a nuclear coordinate window corresponding to the inhomogeneous broadening (taken 170 cm^{-1} (ref. 38) as compared to the total bandwidth of 520 cm^{-1}). The difference between the real and the apparent zero time gradually changes when probing at different nuclear coordinates, but is always less than 50 fs . The phases ϕ (using the real zero) and amplitudes A (normalized to the average amplitude at each wavelength) of the Fourier peak at 75 cm^{-1} , obtained from the simulated curves as described for Fig. 2, are shown in *b* and *c*. The general

dom compared to a purely aqueous environment, this is especially important with respect to dephasing of global motions.

Assignment of oscillations to nuclear vibrations

The origin of the oscillations will be discussed on the basis of data collected at 10 K (Figs 1–5), where they are more clearly visible and the spectral features are better resolved. Figure 1 compares the ground-state absorption spectrum of the region of the lowest electronic transition (P band) with the stimulated emission spectrum measured 10 ps after excitation with a femtosecond pulse centred at 870 nm . The (ground state) absorption band and the (excited state) emission band are well separated (Stokes shift $\sim 500\text{ cm}^{-1}$). This implies that the nuclear modes that are most displaced in the excited state are populated well above the bottom of the excited-state potential energy surface.



features, the increase of the relative amplitude towards the sides of the bands and the apparent reversal of the phase of the oscillations around the bottom of the potential energy well where the amplitude is very small, do not depend on the details of the simulation. These features compare well with a similar analysis of both the 15 and 77 cm^{-1} components of the experimental data of Fig. 2 (*d* and *e*; in *e*, the data of Fig. 5, which show that the bottom of the well is probed near 921.5 nm , are also included). The vertical offset between the simulated and measured curves may be due to very early processes, pumping slightly below the turning point and the uncertainty in zero time, but the sharp π phase-change, together with the disappearance of the amplitude of the oscillations at the maximum of the band, is well reproduced. The asymmetry in the values of the amplitude of oscillations for both frequencies (*e*) is presumably due to the bleaching of the ground state, which adds to the stimulated emission signal at the blue side of the spectrum (Fig. 1) and possibly to interference effects with motion on the ground state.

The transients of stimulated emission are modulated by oscillatory features with main components at 15 and 77 cm^{-1} (Fig. 2). The oscillations are damped in $\sim 2\text{ ps}$; this corresponds to a frequency broadening of $\pm 8\text{ cm}^{-1}$. The amplitudes and phases of the oscillations strongly depend on the detection wavelength. If these oscillations reflect vibrational motion following coherent excitation above the bottom of the excited-state potential surface, a π phase change should be observable at the bottom of the well (Fig. 3). This is precisely what is observed for both oscillatory components. Another observation, the increase of the amplitudes of the oscillating components (relative to the overall signal) towards both sides of the stimulated emission spectrum, is also fully consistent with this hypothesis. As will be discussed later, in the case of oscillations reflecting vibrational motion, a quite different oscillatory pattern is expected when probing the bottom of the well.

FIG. 4 Oscillatory parts of the stimulated emission probed at 930 nm , along with their FT spectra, for different wavelengths of the pump pulses. The oscillatory parts are normalized to the average amplitude of the overall transient absorption traces. Note that the peak of the $\sim 77\text{ cm}^{-1}$ mode shifts to lower frequencies for higher energies of the pump photons.

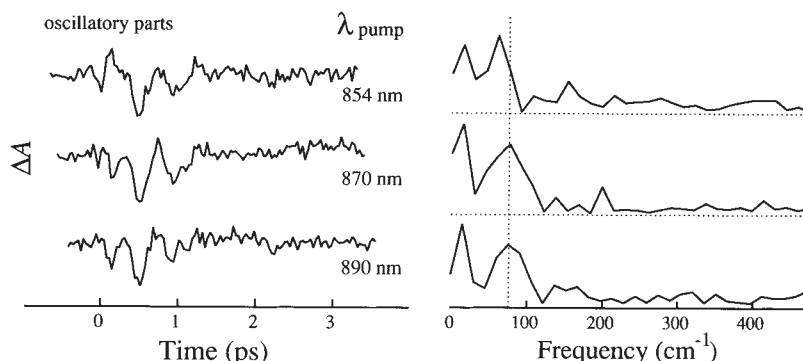
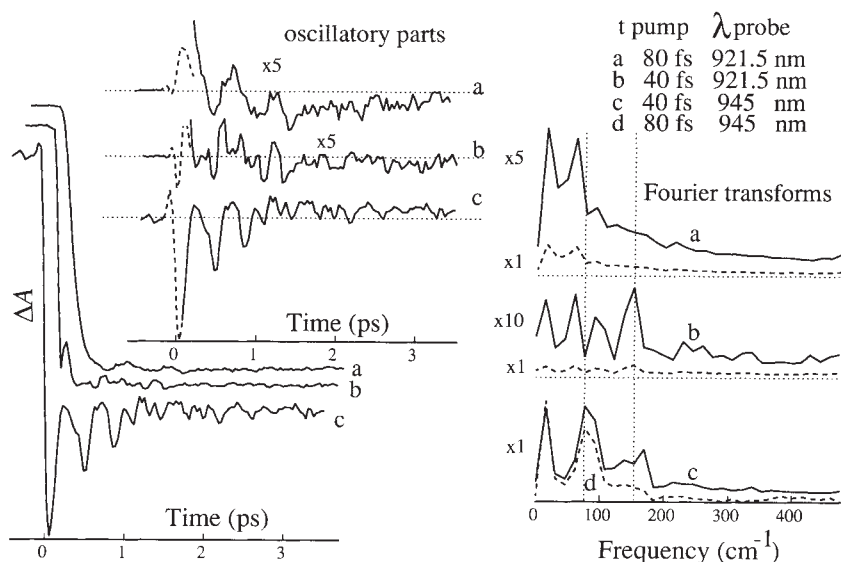


FIG. 5 Oscillations near the bottom of the well, where the second harmonic components of the fundamental frequencies of the modes are expected (see simulations of Fig. 3). 80 fs and 40 fs pump pulses centred at 870 nm were used (see Fig. 1 for pump spectra). The traces are normalized to the average amplitude of the overall signal. In the 40 fs pump pulse experiments the initial peak corresponds to the well known coherence interaction of the pump and probe fields during their time overlap (dotted in the oscillatory parts). As the FT analysis is done on a temporal window beyond the overlap time interval, this does not affect the FT spectrum. Oscillatory parts (after the pump-probe overlap time) and FT (solid lines) are multiplied by 5 for the 921.5 nm traces. The first and second harmonic frequency of the 77 cm^{-1} mode are indicated by dotted lines in the FT spectra. At 921.5 nm, the 15 cm^{-1} peak is strongly diminished as compared to other wavelengths and the 77 cm^{-1} component is absent; some oscillator strength appears at 31 cm^{-1} and a clear peak appears at 61 cm^{-1} (a, b). When 80 fs pump pulses are used, no overtone peak of the 77 cm^{-1} component is observed (a). The use of shorter pump pulses (40 fs) allows the clear observation of this component at 153 cm^{-1} . Its amplitude is about 10% of that of the 77 cm^{-1} mode at 945 nm. Additional peaks at 61 and 92 cm^{-1} are also observed. They correspond, respectively, to the



difference and the sum frequency of the 77 and 15 cm^{-1} modes. Measurements away from the bottom of the well (945 nm) reveal only the two fundamental frequency components and are much less sensitive to the pump pulse duration (c, d).

The initial population of excited-state vibrational levels can be tuned by varying the spectrum of the pump pulse (Fig. 4). For the D_{LL} mutant, a small but significant shift in frequency of the $\sim 77\text{ cm}^{-1}$ feature is observed when scanning the excited-state potential energy surface by about 6 vibrational levels (480 cm^{-1}), suggesting, in a vibrational picture, a slightly anharmonic potential energy surface. We did not detect strong changes in amplitudes of either the 15 or the 77 cm^{-1} features when using a shorter (40 fs instead of 80 fs; compare with Fig. 5) spectrally broader pump pulse, which is consistent with impulsive population of excited-state vibrational modes, but not of ground-state modes²¹. A more complicated dependence on the pump spectra is observed in wild-type reaction centres, which do perform electron transport (not shown).

Apart from the above positive evidence for a vibrational origin of the oscillatory features, the possibility that they reflect electronic quantum beats seems unlikely, because such a mechanism does not predict a phase change at the maximum of the stimulated emission band and cannot explain frequency changes at different pump energies. We conclude that the oscillatory features mainly reflect vibrational motion.

As stated before, very short pulses in resonance with an electronic transition may provoke oscillatory motion of wave packets both in the excited state and, through impulsive stimulated Raman scattering, in the ground state²²⁻²⁴. Selective depletion of the ground state may also provoke oscillatory 'hole' dynamics in the ground state. We base our conclusion that the data presented here do not reflect vibrational motion in the ground state on the following arguments: (1) the expected strong dependence of the oscillation amplitude on the pump pulse duration in the case of an impulsive stimulated Raman process²¹ was not observed; and (2) the extent of the Stokes shift ($\sim 500\text{ cm}^{-1}$, Fig. 1) separates the interrogation zone for excited state of P from that of the ground state (except perhaps when probing at 900 nm). It cannot be excluded, however, that the reported oscillations with similar frequencies in the absorption region¹⁸ contain contributions of ground-state vibrational motion. The absence of impulsive stimulated Raman features in our experiments is consistent with the low resonance-Raman cross-section of the P absorption band compared with that of monomeric bacteriochlorophyll (S. Franzen and A. Shreve, personal communication).

Over and above the assignment of the oscillatory features to nuclear motion on the excited state surface, are the observed frequencies the fundamentals of the activated modes or beatings between higher frequency modes? The possibility of activated modes above 600 cm^{-1} can be excluded in view of the possible excess energy (at most $\sim 600\text{ cm}^{-1}$). Likewise, as the oscillations do not disappear when lowering the excess energy to $\sim 100\text{ cm}^{-1}$ (Fig. 4), they must reflect low-frequency modes and presumably their frequencies correspond to the fundamentals of those modes. This is especially true for the 77 cm^{-1} mode. The lower 15 cm^{-1} vibration might be due to a beating between two modes near 80 cm^{-1} , although the dependence of the oscillation on the probe wavelength (Fig. 3) supports the idea that it directly reflects a 15 cm^{-1} mode.

Probing near the bottom of the potential well

As stated before and illustrated in Fig. 3a, an essentially different oscillatory behaviour is expected when probing the central region of the well as compared to the turning point regions. Here, the wave packet passes twice per vibrational period, in opposite directions, giving rise to oscillations with the double frequencies of the fundamentals. This feature should be most prominent at the bottom of the well, where the fundamental frequencies should vanish. As the dwell time of the wave packet is much shorter in this region, where its momentum is the highest, than at the turning points, the expected amplitude of the second harmonic feature is rather small. Calculations using a crude model predict that in our conditions, the amplitude of the overtone components should be at most $\sim 10\%$ of that of the fundamental frequencies measured near the turning points.

Comparison of the Fourier transform spectra of Fig. 5a and d reveals such a behaviour. As expected, at $\lambda_{\text{probe}} = 921.5\text{ nm}$, near the maximum of the stimulated emission band, the 77 cm^{-1} peak vanishes. Concomitantly, the density of frequency components in the 30 cm^{-1} and 150 cm^{-1} regions increases relatively, although no separate peaks at those frequencies are observed. The appearance of a clear peak at the double frequency of the 15 cm^{-1} mode may be hindered by the appearance of an additional peak at 62 cm^{-1} (discussed below) and by the fact that the 15 cm^{-1} fundamental is essentially damped after one oscillation period. This latter feature precludes the unambiguous Fourier spectral separation of the fundamental from its second har-

monic. The 150 cm^{-1} region is actually outside the vibrational bandwidth determined by the 80 fs pump pulse. Therefore any features in this region are attenuated. To overcome this limitation the pump pulse is shortened to 40 fs by broadening its optical bandwidth (Fig. 1). Figure 5b shows that under these conditions, beside an additional peak at 92 cm^{-1} , which will be discussed below, a clear peak at 153 cm^{-1} is observed. The amplitude of this component is about 10 times lower than that of the 77 cm^{-1} peak at $\lambda_{\text{probe}} = 945\text{ nm}$, and it bears the expected phase characteristics (not shown) of the second harmonic component of the 77 cm^{-1} fundamental. Figure 5c shows that, when probing nearer to the turning point, spectrally broadening the pump pulse does not greatly affect the Fourier transform spectrum in the region below 100 cm^{-1} . Above 100 cm^{-1} , some vibrational oscillator strength becomes apparent, but there is clearly no peak at 153 cm^{-1} , as observed at $\lambda_{\text{probe}} = 921.5\text{ nm}$. These higher-frequency components may reflect coherence of higher-frequency modes, interference effects (see below), or higher-order components related to a non-sinusoidal form of the oscillatory signals.

The vanishing of the 77 cm^{-1} frequency at $\lambda_{\text{probe}} = 921.5\text{ nm}$ further reveals the presence of two small separate bands located at the frequency difference (61 cm^{-1}) and the frequency sum (92 cm^{-1} , attenuated when 80 fs pump pulses are used) of the 15 cm^{-1} and 77 cm^{-1} fundamentals. These features presumably reflect interferences between the two fundamental modes. They can be understood when considering the wave packet motion on a multi-dimensional potential energy surface rather than a cross-section along one mode.

Temperature dependence

The observed damping time of $\sim 2\text{ ps}$ for both the 15 and the 77 cm^{-1} oscillatory features sets a lower limit for the dephasing time of the corresponding vibrational modes. Hence both modes are underdamped at 10 K. This is most apparent for the 77 cm^{-1} mode. For the 15 cm^{-1} feature, damping occurs in about one vibrational period and this mode may be more critically damped. To gain insight into the mechanisms leading to the dephasing of the vibrational modes and to evaluate the relevance of our findings for the functioning of proteins at higher temperatures, the oscillations were recorded as a function of temperature (Fig.

6). On raising the temperature, the amplitude of the modulation gradually decreases in a similar way for both oscillations, although the damping times are not strongly affected. The 77 cm^{-1} Fourier band broadens and a similar effect for the 15 cm^{-1} feature is observed at 290 K. At room temperature (290 K) the oscillations have almost vanished, but a very small but significant modulation of the signal is still observed. It is almost entirely due to the lowest frequency component.

As a general feature, increase in temperature is expected to affect both the preparation of the initial wave packet and efficiency of dephasing through collisional events during the time evolution of the vibrational wave packet (incoherent dephasing). For the former mechanism, the energetic width of the wave packet determines the initial distribution of nuclear coordinates and, in the case of an anharmonic potential, the initial frequency dispersion. The width of the distribution of populated excited-state levels is mainly determined by the pump bandwidth (130 cm^{-1}) at 10 K ($kT = 8\text{ cm}^{-1}$; where k is the Boltzmann constant) and by the ground state Boltzmann distribution at 290 K ($kT = 235\text{ cm}^{-1}$). On warming from 10 K to 290 K, the relative increase of the impulsively populated excited state vibrational levels is very similar: ~ 9 to 16 and ~ 2 to 3, respectively. This may explain the rough similarity of the amplitude decreases for both features. In more detail, the apparent slightly stronger decrease in amplitude for the 77 cm^{-1} mode, as compared to the 15 cm^{-1} mode, may be explained by the vibrational bandwidth of $\sim 70\text{ cm}^{-1}$ in our experiment, which results in amplitude attenuation of the part of the 77 cm^{-1} band that broadens to higher frequencies. A temperature effect on the site inhomogeneity may also affect the oscillatory features. Site inhomogeneous broadening influences the initially prepared wave packet and tends to smear out the oscillations, thus reducing their amplitudes, because of the distribution of nuclear coordinates probed at a particular energy of the electronic transition.

For the time evolution of the wave packet, at higher temperatures we expect a stronger coupling of the low-frequency modes to the bath, which includes the protein moiety and the membrane solvent. Both elastic and inelastic collisions will tend to increase the rate of destruction of the phase relationships within the ensemble of molecules and possibly to overdamp the

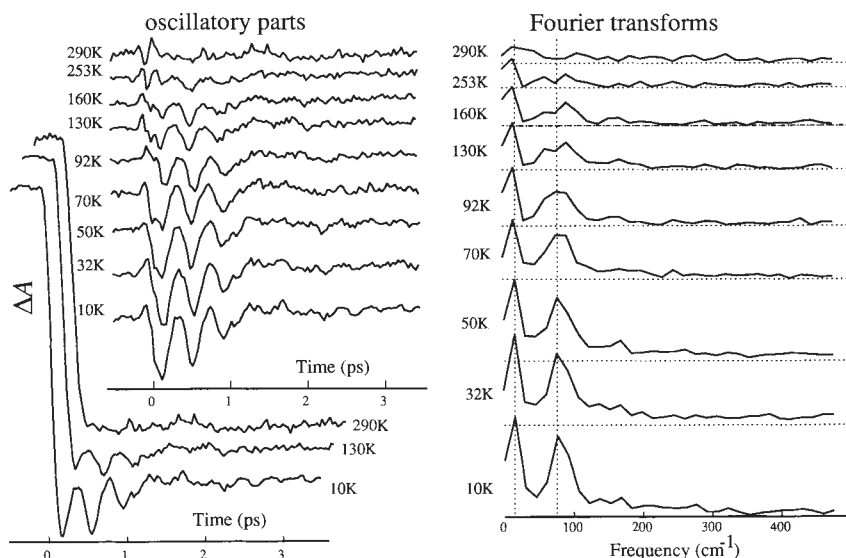


FIG. 6 Temperature dependence of the oscillations detected at 945 nm. Traces are normalized to the average amplitude of the overall signal.

nuclear motions. As a consequence, the wave packets will be broadened and will eventually lose any dynamics. In the experiment this would lead to faster damping of the oscillations. Our results indicate that, whereas the amplitude of the oscillations decreases on warming, the dephasing is not strongly influenced and at all temperatures the motions remain essentially underdamped. Apparently the initial preparation of the wave packet most strongly determines the temperature dependence of the oscillations.

It appears that increasing the temperature up to 290K does not greatly affect the vibrational dephasing through collision-like processes on the timescale of ~ 1 picosecond. The overdamping of modes $< 25 \text{ cm}^{-1}$ at temperatures above 180K, as inferred from neutron scattering data on hydrated water-soluble proteins^{12,24,25} is not observed in the protein complex under study. It is conceivable that the lipid environment of the membrane favours underdamped low-frequency nuclear motion by minimizing the effect of friction provided by a more restricted number of orthogonal degrees of freedom.

The frequency range of the activated modes indicates that they are vibrations of the protein medium^{1,3} rather than intrachromophore vibrational modes; consistently, bacteriochlorophyll monomer vibrational modes below 130 cm^{-1} seem to be absent^{26,27}. The present results reveal only two main underdamped activated modes (15 and 77 cm^{-1}). The presence of only a few low-frequency Raman active modes on excitation in the P absorption region of isolated reaction centres of *Rhodobacter sphaeroides* R-26 (ref. 27) suggests that not many more modes than those two are significantly activated.

Implications for primary electron transfer

The observation that photo-excitation brings about specific con-

certed motions in a protein over a broad range of temperatures suggests a functional role for such motions. This may apply particularly for the transmembrane primary charge separation in functional photosynthetic reaction centres, which occurs on the same timescale of a few picoseconds^{6-8,28}.

The present work suggests that this reaction may occur in a regime where the semi-classic description of electron transfer is no longer valid and where a quantum-mechanical treatment of the nuclear motion will be more appropriate. As mentioned earlier, in conventional treatments it is assumed that the vibrational motions that bring the system to the configuration where the potential surfaces cross are thermally equilibrated (and thus vibrationally dephased) on the timescale of electron transfer. It is also assumed that on average many passages through the crossing region occur before transfer (non-adiabatic limit)^{29,30}. By contrast, it has been argued recently that a mechanism in which vibrational relaxation does not occur before electron transfer is consistent with a range of experimental data⁹. Theoretically, the consequence of both vibrational and electronic coherence on the rate of electron transfer in the condensed phase, including reaction centres, has been explored^{10,11}. A major conclusion of these studies is that under certain conditions near-adiabatic electron transfer from a non-dephased manifold of vibrational levels can be more efficient than in the conventional non-adiabatic regime. Our results show that such a mechanism may apply.

The phase conservation for several oscillatory periods may be a general phenomenon in proteins. But the experimental extension of these studies towards other classes of proteins is not straightforward. The properties of the reaction centre provide the exceptional opportunity to synchronize and visualize nuclear motion with ultrashort light pulses. $\square$

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- Karplus, M. & McCammon, J. A. *Rev. Biochem.* **53**, 263-300 (1983).
- Brooks, B. & Karplus, M. *Proc. natn. Acad. Sci. U.S.A.* **80**, 6571-6575 (1983).
- Go, N., Noguti, T. & Nishikawa, T. *Proc. natn. Acad. Sci. U.S.A.* **80**, 3696-3700 (1983).
- Schulten, K. & Tesch, M. *Chem. Phys.* **158**, 421-446 (1991).
- Warshel, A., Chu, Z.-T. & Parson, W. W. *Science* **246**, 112-116 (1989).
- Martin, J.-L., Breton, J., Hoff, A. J., Migus, A. & Antonetti, A. *Proc. natn. Acad. Sci. U.S.A.* **83**, 957-961 (1986).
- Woodbury, N. W., Becker, M., Middendorf, D. & Parson, W. W. *Biochemistry* **24**, 7516-7521 (1985).
- Fleming, G. R., Martin, J.-L. & Breton, J. *Nature* **333**, 190-192 (1988).
- Middendorf, T. R., Mazzola, L. T., Gaul, D. F., Schenck, C. C. & Boxer, S. G. *J. phys. Chem.* **95**, 10142-10151 (1988).
- Jean, J. M., Friesner, R. A. & Fleming, G. R. *J. chem. Phys.* **96**, 5827-5842 (1992).
- Skourtis, S. S., da Silva, A. J. R., Bialek, W. & Onuchic, J. N. *J. phys. Chem.* **96**, 8034-8041 (1992).
- Smith, J. C. Q. *Rev. Biophys.* **24**, 227-291 (1991).
- Rose, T. S., Rosker, M. J. & Zewail, A. H. *J. chem. Phys.* **91**, 7415-7435 (1989).
- Khundkar, L. R. & Zewail, A. H. *Rev. phys. Chem.* **41**, 15-60 (1990).
- Scherer, N. F., Ziegler, L. D. & Fleming, G. R. *J. chem. Phys.* **96**, 5544-5547 (1992).
- Pollard, W. T., Lee, S.-Y. & Mathies, R. A. *J. chem. Phys.* **92**, 4012-4029 (1990).
- Mathies, R. A., Brito Cruz, C. H., Pollard, W. T. & Shank, C. V. *Science* **240**, 777-779 (1988).
- Vos, M. H. *et al. Proc. natn. Acad. Sci. U.S.A.* **88**, 8885-8889 (1991).
- Breton, J., Martin, J.-L., Lambry, J.-C., Robles, S. J. & Youvan, D. C. in *Reaction Centers of Photosynthetic Bacteria* (ed. Michel-Beyerle, M. E.) 293-302 (Springer, Berlin, 1990).
- Robles, S. J., Breton, J. & Youvan, D. C. in *Reaction Centers of Photosynthetic Bacteria* (ed.

- Michel-Beyerle, M. E.) 283-291 (Springer, Berlin, 1990).
- Pollard, W. T. *et al. J. phys. Chem.* **96**, 6147-6158 (1992).
- Cresnoy, J. & Mokhtari, A. *Phys. Rev.* **A38**, 3566-3576 (1988).
- Yan, Y. X. & Nelson, K. A. *J. chem. Phys.* **87**, 6240-6265 (1987).
- Smith, J., Cusack, S., Tidor, B. & Karplus, M. *J. chem. Phys.* **93**, 2974-2991 (1990).
- Doster, W., Cusack, S. & Petry, W. *Phys. Rev. Lett.* **65**, 1080-1083 (1990).
- Lutz, M. & Mantele, W. in *Chlorophylls* (ed. Scheer, H.) 855-902 (CRC, Boca Raton, 1991).
- Shreve, A. P., Cherepy, N. J., Franzen, S., Boxer, S. G. & Mathies, R. A. *Proc. natn. Acad. Sci. U.S.A.* **88**, 11207-11211 (1991).
- Martin, J.-L. & Vos, M. H. *Rev. Biophys. biomolec. Struct.* **21**, 199-222 (1992).
- Marcus, R. A. & Sutin, N. *Biochim. biophys. Acta* **811**, 265-322 (1985).
- Bixon, M. & Jortner, J. *J. chem. Phys.* **90**, 3795-3800 (1986).
- Deisenhofer, J. P. & Michel, H. *Rev. Biophys. biophys. Chem.* **20**, 247-266 (1991).
- Feher, G., Allen, J. P., Okamura, M. Y. & Rees, D. C. *Nature* **339**, 111-116 (1989).
- Vos, M. H. *et al. Proc. natn. Acad. Sci. U.S.A.* **89**, 613-617 (1992).
- Robles, S. J., Breton, J. & Youvan, D. C. *Science* **248**, 1402-1405 (1990).
- Youvan, D. C., Ismail, S. & Bylina, E. J. *Gene* **38**, 19-30 (1985).
- Bylina, E. J., Robles, S. J. & Youvan, D. C. *Isr. J. Chem.* **28**, 73-78 (1988).
- Breton, J., Bylina, E. J. & Youvan, D. C. *Biochemistry* **28**, 6423-6430 (1989).
- Bowman, R. M., Dantus, M. & Zewail, A. H. *Chem. Phys. Lett.* **174**, 546-552 (1990).
- Johnson, S. G. *et al. J. phys. Chem.* **94**, 5849-5855 (1990).

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Energetic ions at comet Grigg–Skjellerup measured from the Giotto spacecraft

S. M. P. McKenna-Lawlor*, P. W. Daly†, E. Kirsch‡, D. O'Sullivan§, A. Thompson§, K.-P. Wenzel‡ & V. Afonin¶

* Space Technology Ireland, St Patrick's College, Maynooth, Ireland

† Max Planck Institut für Aeronomie, D-3411 Katlenburg-Lindau, Germany

§ Dublin Institute for Advanced Studies, 5 Merrion Square, Dublin, Ireland

‡ Space Science Department, ESA/ESTEC 2200 AG, Noordwijk, The Netherlands

¶ Space Research Institute Profsoyuznaya 84/32, 117810 Moscow, GSP-7, Russia

ON 10 JULY 1992, the Giotto spacecraft flew within about 200 km of the nucleus of comet Grigg–Skjellerup; this is only the third comet at which *in situ* measurements have been made, and the encounter constituted the closest approach so far of a spacecraft to a cometary nucleus. Here we report the detection by the EPONA instrument on Giotto of charged, energetic particles deep within the inner coma of Grigg–Skjellerup. In contrast to previous spacecraft encounters with comets Giacobini–Zinner (in 1985) and Halley (in 1986), well defined, periodic intensity variations recorded in the particle fluxes suggest that the ions close to the nucleus were strongly coupled to the ambient magnetic field. The present data indicate that Giotto flew on the nightside of the nucleus.

A comet nucleus is itself too small to be observed from the Earth, but as it approaches the Sun, mass is lost as an expanding atmosphere composed of dust, molecules, radicals and molecular ions is formed. The molecules undergo complicated chemical reactions in the inner regions of the cometary coma. Further out, ions are created from cometary neutrals through photoionization and other mechanisms.

These newly formed ions are created essentially at rest (velocity $\sim 1 \text{ km s}^{-1}$) in the comet's rest frame, where they undergo cycloidal motion in the crossed electric and magnetic fields of the solar wind (the pick-up process). Their peak energy is then $E_{\text{max}} = 4A \sin^2 \theta E_{\text{sw}}$ (where A is ion mass in atomic mass units, θ is the angle between the solar wind velocity vector and the magnetic field direction, and E_{sw} is the energy of a proton travelling at the solar wind bulk speed).

In the rest frame of the solar wind, the ions are created with a speed equal to the solar wind speed in the comet's rest frame, and form a ring distribution in velocity space as they gyrate about the magnetic field lines. Such a distribution is highly unstable and gives rise to resonant ion-cyclotron waves, making the magnetic field turbulent. Wave scattering in this turbulent field acts to make the ion distribution isotropic, by producing a shell-like distribution of particles (pitch angle scattering) within a few gyroperiods. Further shells of lower energies are similarly progressively formed. If efficient pitch angle scattering occurs in the flow rest frame, then the peak energy (E_{max}) of the ions is $4AE_{\text{sw}}$, independent of the magnetic field direction, and the mean direction of motion depends only on the direction of the solar wind flow.

The energy and momentum imparted to the ions thus picked up comes from the solar wind which, as momentum is conserved, is decelerated by ever-increasing amounts as the comet nucleus is approached (mass loading effect). When the number

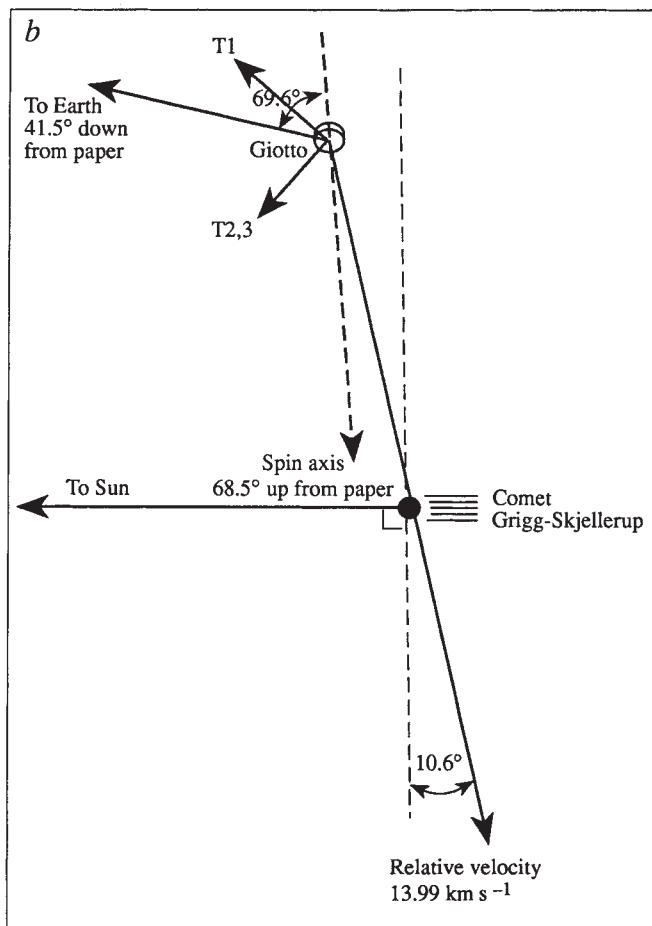
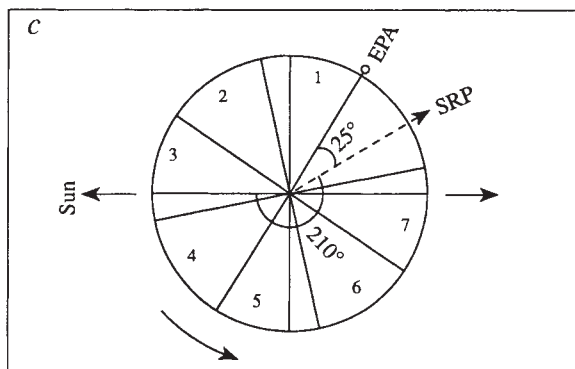
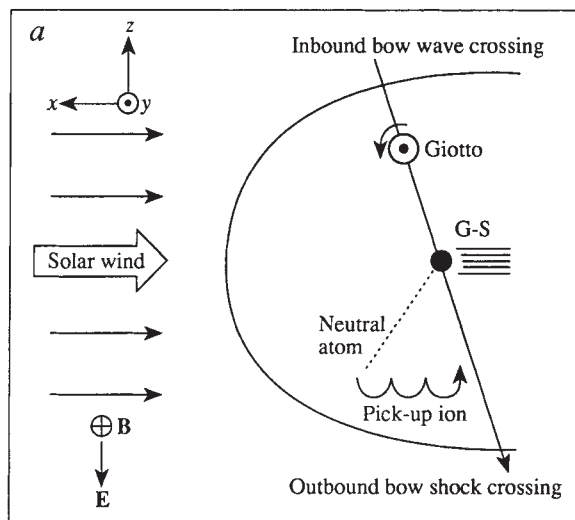
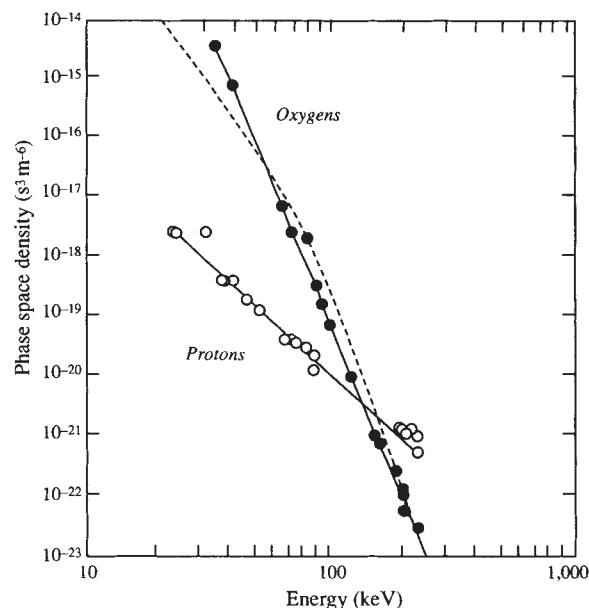


FIG. 2 Spectra fitting the counts recorded by EPONA, from 12.50–12.55 UT (1992 July 10), to a mixture of protons and oxygens. Species separation has been done by nonlinear least-squares fitting to two independent power-law spectra transformed into the solar wind frame (phase-space density is the number of ions per unit volume per unit velocity³). The solid lines are the resulting power-law spectra. The dotted line is a theoretical spectrum for second-order Fermi acceleration of oxygen ions³, taking into account the effect of adiabatic compression in the cometary accretion flow and assuming that the mean free path corresponding to random scattering by ambient waves is of the order of 30 gyroradii.



density of the pick-up ions approaches $\sim 1\%$ of the number density of the solar wind, a weak shock forms and the frozen-in interplanetary magnetic field starts to drape around the comet to form an elongated, induced magnetotail¹.

Here we present energetic ion measurements made by the EPONA instrument on board Giotto during the encounter with comet Grigg-Skjellerup (Fig. 1) on 10 July 1992. EPONA features three semiconductor telescopes (T1, 2 and 3), each with geometric factor $2.0 \times 10^{-2} \text{ cm}^2 \text{ sr}$. We will only describe the data from T1 and T3. Ionized particle energies are determined by measuring their energy loss in the solid-state detectors in various energy intervals². The (calibrated) energy thresholds in channels 1–4 of T1 and T3 are 29, 44, 78 and 217 keV for protons and 60, 97, 144 and 260 keV for H_2O^+ ions. Details of the encounter geometry, the telescope look directions and the sectors into which data are divided are shown in Fig. 1.

◀ FIG. 1 *a* Schematic representation of the trajectory of the Giotto spacecraft through the reported⁴ bow wave and bow shock (BW, BS) of comet Grigg-Skjellerup (G–S). A bow shock, defined as “a relatively steep change in the magnetic field vector components from outer to inner states that show little linear trend compared with the transition itself” was identified in ref. 4, outbound at $25 \times 10^3 \text{ km}$ from closest approach (CA). In the absence of a corresponding jump in the magnetic field vectors between two relatively uniform regions, or of a plasma shock without such a jump in the magnetic field data, it was inferred in ref. 4 that a cometary bow wave was traversed, inbound, at $20 \times 10^3 \text{ km}$ from CA. The coordinate system is such that x points towards the Sun and y lies in the ecliptic plane. Neutral atoms ejected from the comet move at low speeds through the solar wind ($\sim 1 \text{ km s}^{-1}$) until ionized by photon and/or electron fluxes. The resulting ions, being subject to the electric and magnetic fields that drive the solar wind ions, are compelled to move with them in cycloidal orbits. Hence they are said to be ‘picked-up’ by the solar wind. *b*, Encounter geometry and look directions of the three EPONA telescopes. T1 is oriented at 45° , and T2 and T3 both oriented at 135° to the spacecraft spin axis. T1 viewed in the backward hemisphere relative to the spacecraft’s inbound flight path; T2 and T3 viewed in the forward hemisphere. The angle between the spacecraft spin axis and the relative velocity vector was 68.5° during the encounter. Measurements during the 4-s rotation period of the spacecraft were divided into eight sampling intervals (*c*), each of 0.5 s duration (S1–S8), T1, taking the odd-sector measurements and T2 and T3 the even-sector measurements (EPA represents the position of EPONA on the spacecraft, SRP denotes the Sun reference pulse).

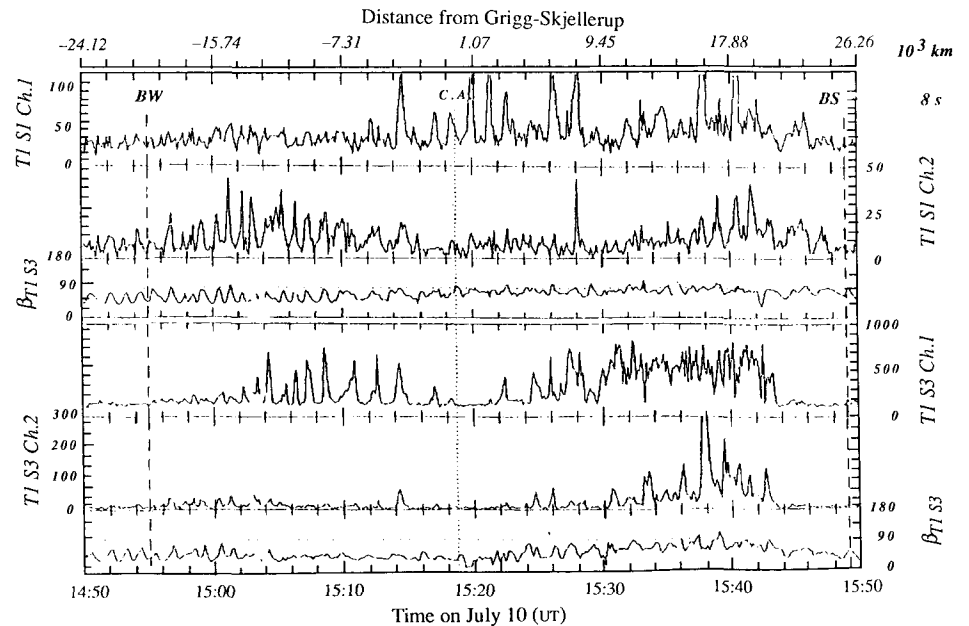
All the observations at the comet are expressed in spacecraft event time. The first small signature of cometary ions (not illustrated) was detected from $\sim 03:10 \text{ UT}$ on 10 July in T1, sector S5 channels 1–4 (distance from closest approach $\sim 6 \times 10^5 \text{ km}$), and gradually increased. In general, during the encounter, the fluxes recorded were higher by a factor of 3–10 in sector S3 of T1 than in sectors S1, 5 and 7. The flux before encounter was also anisotropic, showing that there was a Sun-related contribution in T1, S3. Cometary ions (outbound) faded at $\sim 20:50 \text{ UT}$ in T1, S3 channels 1–4 (distance from closest approach $\sim 6 \times 10^6 \text{ km}$).

Figure 2 presents a pair of typical spectra for the period 12.50–12.55 UT. Protons and oxygen ions have been separated out by a nonlinear least-squares fitting method that reproduces the measured count rates from the sum of two independent power-law spectra transformed into the solar wind frame. Although it is not possible to separate protons of cometary origin from those in the solar wind, we expect the latter to dominate. The oxygens are interpreted to be pick-up ions of cometary origin, as the corresponding solar wind component is likely to be small.

The maximum pick-up energy at the prevailing solar wind speed during encounter (about 400 km s^{-1} as reported by the Johnstone Plasma Analyzer Experimenters, personal communication) was 3.32 keV for a proton and 52 keV for a water group ion. Such particles would fall below the EPONA detection threshold. As the particles recorded by EPONA showed energies of $\geq 260 \text{ keV}$, some additional accelerating mechanism(s) must have been operating. Wave scattering can result in energy diffusion through second-order Fermi acceleration. This is caused by stochastic ion scattering in the presence of strong wave turbulence. The dotted line in Fig. 2 is a theoretical spectrum for second-order Fermi acceleration of oxygen ions³ and provides a reasonable qualitative fit to the experimental data.

Figure 3 shows the count rates recorded by EPONA in T1 sector S1 and T1 sector S3 (channels 1, 2) between the times that Giotto crossed the reported⁴ bow wave (inbound) and bow shock (outbound) of the comet⁴. Plotted against the count rates in Fig. 3 are the corresponding instantaneous pitch angles, β , between the velocity vector of the ion and the magnetic field direction. Oscillations in the magnetic field vector, reflected in the pitch angle variations, have a period of about 1 minute, indicating the presence of oxygen cyclotron waves (singly charged oxygen ions in a field of 20 nT have a cyclotron

FIG. 3 Count rates measured by EPONA in T1, S1 and T1, S3, energy channels 1 and 2, predominantly between the inbound bow wave (BW) and outbound bow shock (BS) crossings, with corresponding instantaneous pitch angles (β) plotted against time (β is the angle between the velocity vector of the ion and the magnetic field direction). The time of closest approach to the comet (CA) is indicated.



frequency of 52 s). The particle count rates were highest when the measuring sector was almost perpendicular to the magnetic field ($\beta = 90^\circ$). Oscillations in the particle intensities are interpreted as being due to the stable pitch angle distribution moving with the magnetic field periodically through the view direction of the T1 detector.

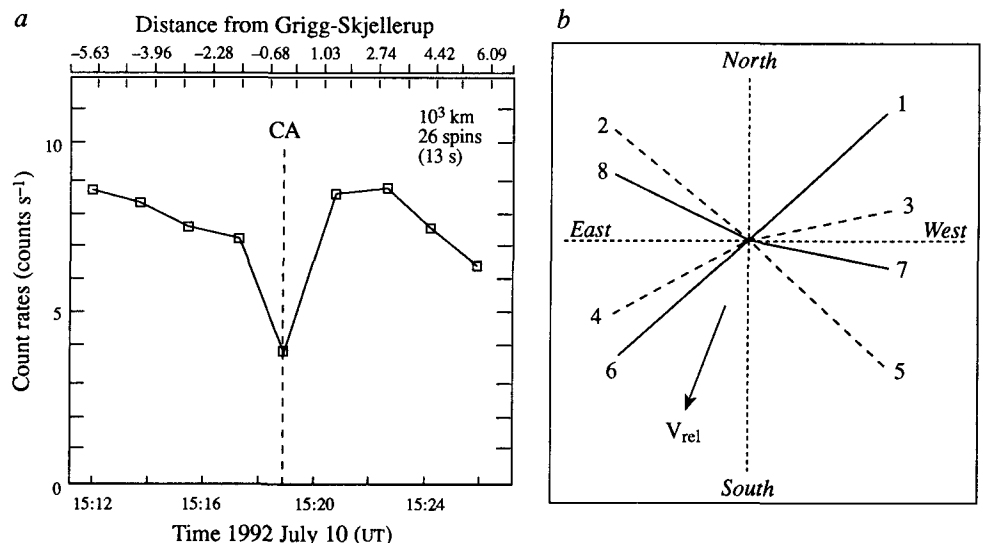
Figure 4a shows particle counts from T3 sector S4, channel 2, averaged over 26 spins (13 s accumulation time per point) spanning closest approach. The number of counts in the central point is 48, and on either side 94 and 107. The drop in counts (observed only in T3, S4) represents 5 standard deviations. If the reduction in flux was caused by absorption along the particle's path, then the occulting obstacle was east-southeast and sunward of the spacecraft (the look direction of sector 4; see Fig. 4b). As the estimated closest distance to the comet nucleus (200 km, ESOC tracking data) is much less than the 2,000 km gyroradius of the oxygen ions, gyrocurvature may be neglected in determining the direction of the obstacle. The size of the particle data gap is $\sim 1,500$ km (100 s times the 14 km s^{-1} flyby

speed). The distorted magnetic field around the comet nucleus could have constituted the 'occluding object'.

Grigg-Skjellerup, with a nuclear gas production rate of $Q = 6 \times 10^{27} \text{ s}^{-1}$, is only the third comet at which *in situ* energetic particle measurements have been made⁵ — the others were the highly active Halley ($Q = 6.9 \times 10^{29} \text{ s}^{-1}$) and less active Giacobini-Zinner ($Q = 2 \times 10^{28} \text{ s}^{-1}$). The Giotto encounters with Grigg-Skjellerup and Halley, and the encounter of the International Cometary Explorer (ICE) with Giacobini-Zinner, occurred at roughly the same Sun/comet separation, so the effects of radial variations in the solar wind and solar radiation can essentially be disregarded when comparing these data. The closest approach of Giotto to comet Halley and of ICE to Giacobini-Zinner occurred, respectively, at 600 km to sunward and at 7,800 km to tailward of the nucleus concerned. The EPONA observations suggest that Giotto flew on the nightside of the nucleus of Grigg-Skjellerup (targeted miss distance 200 km).

In all three cases, large fluxes of pick-up ions were recorded

FIG. 4 a, Count rates measured by EPONA in T3, channel 2, S4 averaged over 26 spins (accumulation time per point 13 s). The scale at the top of the figure gives the distance in 10^3 km before and after closest approach (CA). b, Geometry of the flyby in a plane perpendicular to the ecliptic (the east-west axis) and the comet-Sun line, looking towards the Sun. V_{rel} is the direction of the velocity of Giotto relative to Grigg-Skjellerup. The numbered lines represent the projection of the EPONA look directions onto the page for the corresponding sector: solid lines looking out of the page, dashed lines into it. The shorter the line, the further the look direction is away from the plane of the page.



far upstream of the bowshock, streaming predominantly away from the Sun^{6,7}. The fluxes measured by EPONA at Grigg-Skjellerup were almost a factor of 100 lower than those at Halley⁷. However, the particle energies at all three comets were similar (≥ 260 keV) and always significantly exceeded the maximum energy attainable by the pick-up process alone.

The energetic particle profiles recorded at Grigg-Skjellerup were markedly different from those detected during previous encounters. In particular, the variations in fluxes at the oxygen ion cyclotron frequency recorded close to the nucleus have not

been seen before. □

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1. *Comets in the Post-Halley Era* (eds Newburn, R. L. Jr, Neugebauer, M. & Rahe, J.) (Kluwer, Dordrecht, 1991).
2. McKenna Lawlor S. et al. *J. Phys. E. Scient. Instrum.* **200**, 732–740 (1987).
3. Ip, W. H. & Axford, W. I. *Planet. Space Sci.* **34**, 1061–1065 (1986).
4. Neubauer, F. M. et al. *Astr. Astrophys.* **261**, L5–L8 (1993).
5. Coates, A. J. et al. *Geophys. Res. Lett.* **20**, 483–486 (1993).
6. Hynds, R. J. et al. *Science* **232**, 361–365 (1986).
7. McKenna-Lawlor, S. M. P. et al. *Icarus* **87**, 430–439 (1990).

Detection of the hydroxyl radical in the Saturn magnetosphere

D.E. Shemansky, P. Matheson, D.T. Hall*, H.-Y. Hu & T.M. Tripp†

Department of Aerospace Engineering, University of Southern California, Los Angeles, California 90089-1191, USA

* Department of Physics, Johns Hopkins University, Baltimore, Maryland 21218, USA

† Department of Astronomy, University of Wisconsin, Madison, Wisconsin 53706, USA

THE magnetosphere in the vicinity of the orbits of Saturn's icy satellites consists of a low-density plasma, in which the electrons are an order of magnitude cooler than the accompanying heavy ions¹. Most models^{2–12} neglect this fact, even though radiative cooling and diffusive loss rates are both too slow to account for the observed temperatures. Shemansky and Hall¹³ have recently proposed that the electrons could be cooled by the presence of a large abundance of neutral gas, derived mainly from the breakdown products of H₂O (mainly O and OH) from the icy satellites. Hydrogen radicals have been reported in this region¹³, but these originate from the atmosphere of Saturn itself; no satellite-derived neutral species have been detected. Here we report the detection of neutral OH molecules near the orbit of Tethys, using the Faint Object Spectrograph on the Hubble Space Telescope. Our results suggest that neutral OH is one of the dominant species in Saturn's inner magnetosphere, implying a source rate for H₂O twenty times greater than current theoretical estimates^{5,6}. One possible explanation is that the micrometeorite erosion rates of the inner satellites are significantly higher than expected.

Spectra of Saturn's magnetosphere were obtained in two sets of exposures on 24 and 26 August 1992. The observations were made from the dark Earth atmosphere, along a line of sight passing through the satellite plane and displaced by 4.5 Saturn radii (R_S) from the centre of Saturn on the local dusk side. This location coincides with a maximum in the abundance of magnetosphere plasma, as modelled¹ from Voyager spacecraft observations, and was expected to correspond to a maximum in neutral gas abundance¹³. The gas was assumed to be in stable orbit around the planet, and the dusk side of the planet was selected for observation because positive heliocentric radial velocities (that is, velocities away from the Sun) provide a maximum scattering efficiency for solar radiation. The scattering of solar radiation is expected to be the chief emission process in this case, just as it is in comet atmospheres. The line of sight was clear of satellites, so could be acquired entirely from the magnetospheric gas. A spectrograph aperture was selected for maximum throughput limited by a desired wavelength resolution ($\lambda/\Delta\lambda$) of at least 600. The total exposure time for the two days of observations was about 3.5 hours. The dispersive dimension of the selected aperture was 4.4 Å. Other observational details are given in Fig. 1. The spectra obtained on the two

days showed no detectable differences, and were combined to minimize signal noise. The calibrated spectrum was produced using the standard procedure of flat fielding and subtraction of sky background before calibration. Figure 1 shows the spectrum, which includes a substantial component following the shape of the solar spectrum. Such a component is expected because of sunlight scattered from the rings of Saturn, which were near maximum inclination and only 20 arcsec from the instrument's field of view. The signal in excess of the scattered solar spectrum near 3,085 Å is attributed to OH.

The solar spectrum has been accurately measured at high resolution¹⁴ at wavelengths longer than 2,960 Å. This spectrum has been used to predict the spectrum of OH emission in radiative equilibrium. Models calculated on this basis have accurately matched comet spectra¹⁵, and we have used our current code to match the spectrum obtained by the International Ultraviolet Explorer for comet Halley (T.M.T., D.E.S., D.T.H. and T.H.M., manuscript in preparation). Figure 1 shows the synthesized solar spectrum, scaled to match the observed spectrum in the wavelength intervals 3,000–3,030 Å and 3,120–3,150 Å. The difference spectrum plotted in Fig. 1 shows excess emission near 3,085 Å matched by the OH band emission model. The OH model combines solar and electron excitation processes in a statistically equilibrated system. Solar fluorescence dominates, and deleting the plasma excitation has negligible effect. The observed and synthesized spectra show a good match to the main feature, the A–X (0,0) band. Other possible features are at the noise level. The OH gas orbiting at 4.5 R_S has a bulk velocity vector of +10.4 km s⁻¹ along the line between the Sun and the point of observation. The brightness of the OH (A $\Sigma^+ + X^2\Pi$) (0,0) band determined from the model fit to the data is 37 ± 12 Rayleighs (R). Given the known solar flux¹⁴ and the plasma parameters in Table 1, our calculated value for the excitation probability of the OH band is 4.1×10^{-6} s⁻¹. With the spatial parameters of ref. 13, this translates to a mean density of 160 ± 50 molecules cm⁻³.

Table 1 compares observations and theoretical calculations relevant to the present results. We summarize previous observations of neutral gas here. Consideration of the physical chemistry of H₂O extracted from the icy satellites predicts the presence of H, O, OH and other products indicated in Table 1. The hydroxyl radical has not previously been observed. Atomic hydrogen was first detected in 1975¹⁶ and confirmed by later observations. It was generally assumed that the hydrogen was an escape product from the Titan atmosphere. The lack of spatial resolution in the earlier measurements did not allow confirmation of the location of the source. Observations obtained by the Voyager Ultraviolet Spectrograph System (UVS) provides better spatial definition, but the first reported results¹⁷ were based on a limited subset of the data. The distribution was described as a torus with an inner radius of $\sim 8 R_S$, the inner region being essentially devoid of neutral gas (see Table 1). Shemansky and Hall¹³, in an extensive analysis of the Voyager UVS data, discount this result, and claim that the inner magnetosphere in fact contains most of the gas in the system, with increasing densities of H merging into the planet atmosphere. The initial description¹⁷ is, according to the analysis of

TABLE 1 Observed and modelled magnetosphere populations

Observations at ~ 5 R _s	Date	Method	[H]	[OH]	[H ⁺]	[O ⁺ (OH ⁺)]	Comment
Broadfoot <i>et al.</i> ¹⁷	1981	Voy UVS*	0				'H'-cavity†
Richardson and Sittler ¹	1981	Voy PLS‡			2.8	29	Peak values increases inward
Shemansky and Hall ¹³	1981	Voy UVS§	100				
Current	1992	HST-FOS		160			
Models	$\dot{N}_{H_2O}$	$\dot{N}_H$	[H]	[O(OH)]	[H ⁺]	[O ⁺ (OH ⁺)]	Transport time (days)
Eviatar ⁴	1¶						~ 900
Richardson <i>et al.</i> ⁷ #	1	0.23	6.1	2.2 (2.4)	2.1	3.4 (1.9)	∞
Richardson and Eviatar ⁸	1**	~ 0.4	8	9	2.5	9.5	∞
Richardson and Eviatar ⁹	1††	1††			3	15‡‡	∞
Johnson <i>et al.</i> ⁵ §§	0.7	1		~ 5	~ 2	~ 25	
Barbosa ¹⁰	1	10	6			~ 25	30
Shemansky and Hall ¹³	28	0	136	414 (35.5) ¶¶	3.29	24.5 (1.05)	40
Richardson ¹²	1.0		5		~ 2 §§	~ 25 §§	5,400
Current ##	24.8	12.6***	100	199 (160)	3.15	19.5 (4.51)	30

All local densities are in cm⁻³. [O⁺ (OH⁺)] and [O(OH)] represent total heavy component unless both O and OH and/or O⁺ and OH⁺ are specified. $\dot{N}_{H_2O}$ is the total heavy neutral source ($\times 10^{26}$ s⁻¹) and $\dot{N}_H$ the total hydrogen source ($\times 10^{26}$ s⁻¹).

* Summed scans of H Ly- α . Poor coverage of inner region.

† We consider this result to be erroneous.

‡ Voyager Plasma Science Experiment; heavy ion components from water are mutually indistinguishable.

§ Reanalysis of Voyager H Ly- α scans. Complete coverage of area.

|| Source brightness of 37R in OH 3,085 Å band.

¶ Ring source. Only 10^{24} s⁻¹ ions produced in Dione–Tethys Region.

Results of model calculations at 5 eV.

** Inferred rate.

†† Inferred from model's timescale, Fig. 8.

‡‡ Heavy ion is assumed to be H₂O⁺.

§§ Approximate average equatorial values shown. Ion data are from PLS model.

||| Inferred from Fig. 4 of paper.

¶¶ OH photodissociation rate is 5.6 times too high.

Cold electron density and temperature [e_c] = 31.4 cm⁻³ and [T_{ec}] = 4 eV. Hot electron density and temperature [e_h] = 0.4 cm⁻³ and [T_{eh}] = 120 eV.

*** Implied rate. Calculation held [H] = 100 cm⁻³ fixed.

ref. 13, not supported by the reduced data set. The observed atomic hydrogen is latitudinally extensive^{13,18} and longitudinally non-uniform¹³, indicating that its source is Saturn's atmosphere¹³. Atomic hydrogen from the icy satellites has never been observationally identified, primarily because of masking by the source at Saturn. Atomic oxygen has not been detected, because it does not efficiently scatter solar radiation.

Theoretical calculations predicting the content of the inner magnetosphere are summarized in Table 1. Of these, only ref. 13 tolerates more than a very small population of neutral gas. Consequently, the idea of substantial amounts of H in the inner region has essentially been rejected^{2-12,19}. The large abundance of OH reported here also runs counter to the reports summarized in Table 1, with the exception of ref. 13. In fact, the observed abundance of OH is larger than predicted in ref. 13.

We find, however, that the rate coefficient for the photodissociation of OH used there was 5.6 times too high, and resulted in an underestimate for OH density. Corrected values appear in the last row of Table 1. This updated model predicts that atomic oxygen should be the dominant species.

The strong disagreement between the observations of substantial amounts of neutral gas and most published theories of the inner magnetosphere does not have a simple explanation and cannot be fully addressed here. In the principal theories²⁻¹² neutral gas abundance is limited by the restriction in the mass loading rate of the plasma, either through calculated limits on source rates at the icy satellites, or through assumed limitation on the diffusive loss of plasma from the system. In reality the large neutral abundance controls the loss of plasma through charge exchange. Most recent calculations^{10,11,12,19} use a com-

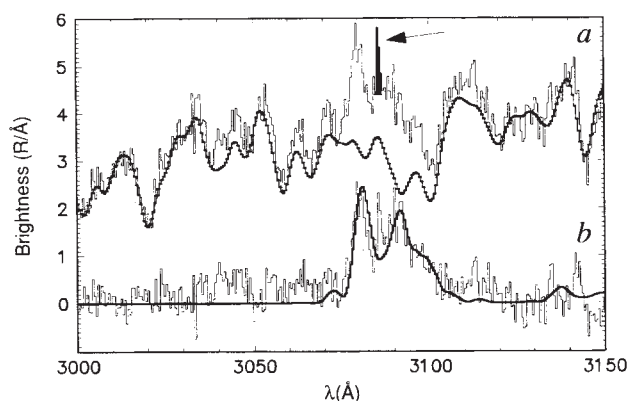


FIG. 1 *a*, Accumulated reduced FOS spectrum of ~ 3.5 hours integrated observation time, shown in the region of the OH A–X (0,0) band near 3,085 Å (light line). A solar model (heavy line) convolved with the instrument transmission function and scaled to the observation is superposed. Solar photons are scattered into the instrument from the nearby rings. Signal contamination from incomplete fixed pattern noise correction is indicated by the arrow. *b*, Difference spectrum along with an OH model spectrum (heavy line). The unambiguous presence of OH is manifest by the A–X (0,0) band emissions near 3,085 Å. No other features in the difference spectrum are identified above the noise level. The observations were obtained using the FOS red detector and G270 grating, which covers the approximate range 2,000–3,200 Å. A spectral resolution of 4.4 Å was achieved using the 0.7 × 2-bar (C-4) aperture. The telescope was pointed 4.5 R_s from Saturn's centre on the dusk side, to coincide with the observed maximum in plasma abundance. The exposures were obtained in the night sky on 24 and 26 August 1992. No satellites were in the field of view during the exposures. The data from each day were accumulated into the single reduced spectrum shown above. The source brightness in the OH band is 37 ± 12 rayleighs. Ordinate unit R/Å is in rayleighs per angstrom.

mon limit on source rate established by the calculations of Pospieszalska and Johnson⁶, although other differences exist between them. The source in this case is limited to the icy satellites. The ring system is excluded as a possible source because a prohibitively large average kinetic energy (25 eV per molecule) is required to deliver the H₂O to the orbital location of the observation. The model used in ref. 13, which resulted in the prediction of large neutral abundances of H₂O products, differed from the other works in two respects. First, source and diffusive loss characteristics were dictated by the measured plasma parameters such as ion partitioning. Second, an approximate balance in the energy budget was required as part of the calculation. It is argued that the measured temperature of the ambient electrons (~4 eV) can only be explained by the cooling mechanism provided by a relatively high density of neutral gas. The calculations of ref. 13 and the current correction of those results essentially agree with the observations (Table 1).

The total estimated source rates can be obtained from a volumetric partitioning code¹³ (last entry, Table 1). The hydrogen number density is fixed at $[H] = 100 \text{ cm}^{-3}$ to correspond to the hydrogen abundance observed by Voyager 1. The source is considered to be a mix of water and oxygen, where the oxygen source represents a population of satellite gas formed from dissociation of water while still in the satellite's tenuous atmosphere, or near its surface. The total source rate is calculated for a total gas volume of $3 \times 10^{31} \text{ cm}^3$, which corresponds to the annular volume defined by the orbits of the satellites Enceladus and Rhea and the region $\pm 0.25 R_S$ out of the equatorial plane. The calculation assumes a mass-dependent diffusive loss time in order to reduce the proton content to the value reported for the Voyager plasma¹. The total source rate remains about $2 \times 10^{27} \text{ s}^{-1}$ as calculated by ref. 13, or about 20 times higher than those proposed by the other models. Large amounts of OH imply equally large amounts of neutral O. The partitioning of $[O^+]$, $[OH^+]$ and $[H_2O^+]$ varies with the relative source rates of O and H₂O.

The large source rate required by the observations may force us to reassess our understanding of Solar System structure. Water products in the magnetosphere have been assumed to be produced when magnetospheric plasma sputters H₂O molecules off the icy satellite surfaces. If this source is inadequate, a plausible explanation may be that primordial material in the form of micrometeorites contributes to the sputtering. The implication is that the flux of these particles is much larger than previously believed.

The measured abundances indicate that the local plasma electron temperature and hence rate processes in the system are determined by the quenching effect of the neutral gas¹³. This is in sharp contrast to the plasma conditions at Jupiter. A general expectation for an internally sustained plasma torus controlled by radiative loss is that it relaxes to a dominantly neutral gas²⁰. However, the ratio of neutral number density of electron density ($[N]/[e]$) in the equatorial region of Saturn's magnetosphere near $4.5 R_S$ is of order 10, whereas in the Jovian Io plasma torus $[N]/[e] \approx 0.02$. This suggests that there are fundamental differences in energy source mechanisms for the two systems (see ref. 20), with Jupiter's plasma torus apparently requiring an external energy source. The large source rate of mass at Saturn indicates that it may be possible to observe OH in the Jupiter system, particularly near the orbit of Europa, where increased amounts of oxygen have been reported²¹. □

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1. Richardson, J. D. & Sittler, E. C. *J. geophys. Res.* **95**, 12019–12031 (1990).
2. Ip, W. H. *Astrophys. J.* **246**, 344–353 (1981).
3. Sieveka, E. M. & Johnson, R. E. *Icarus* **51**, 528–548 (1982).
4. Eviatar, A. *J. geophys. Res.* **89**, 3821–3828 (1984).
5. Johnson, R. E. *et al. Icarus* **77**, 311–329 (1989).
6. Pospieszalska, M. K. & Johnson, R. E. *Icarus* **93**, 45–52 (1991).
7. Richardson, J. D., Eviatar, A. & Siscoe, G. L. *J. geophys. Res.* **91**, 8749–8755 (1986).
8. Richardson, J. D. & Eviatar, A. *Geophys. Res. Lett.* **14**, 999–1002 (1987).

9. Richardson, J. D. & Eviatar, A. *J. geophys. Res.* **93**, 7297–7306 (1988).
10. Barbosa, D. D. *J. geophys. Res.* **95**, 17167–17177 (1990).
11. Eviatar, A. & Richardson, J. D. *Ann. geophys.* **8**, 725–732 (1990).
12. Richardson, J. D. *J. geophys. Res.* **97**, 13705–13713 (1992).
13. Shemansky, D. E. & Hall, D. T. *J. geophys. Res.* **97**, 4143–4161 (1992).
14. Kurucz, R. L., Furenlid, I., Brault, J. & Testerman, L. *Solar Flux Atlas for 296 to 1300 nm* (Harvard Univ. Press, 1984).
15. Schleicher, D. G. & A'Hearn, M. F. *Astrophys. J.* **331**, 1058–1077 (1988).
16. Weiser, H., Vitz, R. C. & Moos, H. W. *Science* **197**, 755–757 (1977).
17. Broadfoot, A. L. *et al. Science* **212**, 206–211 (1981).
18. Hilton, D. A. & Hunten, D. M. *Icarus* **73**, 248–268 (1988).
19. Eviatar, A. & Richardson, J. D. *Ann. geophys.* **10**, 511–518 (1992).
20. Shemansky, D. E. *J. geophys. Res.* **93**, 1773–1784 (1988).
21. Bagenal, F., Shemansky, D. E., McNutt, R. L., Jr, Schreier, R. & Eviatar, A. *Geophys. Res. Lett.* **19**, 79–82 (1992).

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Origin of luminescence from porous silicon deduced by synchrotron-light-induced optical luminescence

T. K. Sham, D. T. Jiang, I. Coulthard, J. W. Lorimer, X. H. Feng, K. H. Tan, S. P. Frigo*, R. A. Rosenberg†, D. C. Houghton‡ & B. Bryskiewicz‡

Department of Chemistry, The University of Western Ontario, London, Ontario N6A 5B7, Canada

*Synchrotron Radiation Center, University of Wisconsin-Madison, Stoughton, Wisconsin 53589, USA

†Advanced Photon Source, Argonne National Laboratory, Argonne, Illinois 60439, USA

‡Institute for Microstructural Science, National Research Council, Ottawa, Ontario K1A 0R6, Canada

FOLLOWING reports of intense optical luminescence from porous silicon^{1,2}, the opportunity for engineering optoelectronic devices using this material^{3,4} has attracted considerable attention. At present, however, the question of the origin of the luminescence has not been fully resolved⁵. The quantum-confinement model^{6–8} suggests that a quantum size effect gives optical transitions, and hence luminescence, in the visible range — this idea gains support from the wavelength dependence of the luminescence on porosity. An alternative model^{9,10} attributes the luminescence to siloxene-like compounds¹¹ formed on the silicon surface. A third model, which invokes hydrogenated amorphous silicon as a possible source^{12,13}, seems to be contradicted by X-ray absorption fine structure (XAFS) studies^{14–16}. Here we report optical luminescence in porous silicon and siloxene induced by soft X-rays with energies near the silicon K-edge (1,839 eV). Using the luminescence together with the total electron yield, we can obtain the XAFS spectra for the luminescent sites in both materials. Our results show that the luminescence from porous silicon does not derive from siloxene (either freshly prepared or annealed), and thus suggest that the quantum-confinement model seems to provide the only viable explanation.

Previous work on synchrotron-radiation-induced luminescence near an absorption threshold of a core level^{18–24} has shown that the optical luminescence yield depends strongly on the site and chemical environment of the absorbing atom¹⁸. The potential of XAFS¹⁹ to shed light on the structural and electronic properties of porous silicon has already been demonstrated^{14–16}. Here we attempt to determine whether the luminescence can be ascribed to quantum confinement or to siloxene, using XAFS combined with X-ray excited optical luminescence (XEOL)^{18,20–22} at the Si K-edge. The total optical photon yield is recorded as a function of soft X-ray energy across

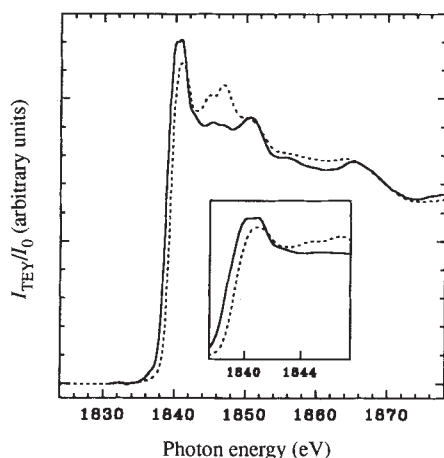


FIG. 1 Room-temperature TEY spectra of porous silicon (dashed line) and Si(100) (solid line) near the Si K-edge.

the Si K-edge. Under favourable experimental conditions²³, the spectrum should represent the absorption spectrum of the Si site which is primarily responsible for the optical luminescence. Porous silicon shows optical luminescence at room temperature when excited by X-ray photons, whereas crystalline Si(100) does not²⁴.

Porous silicon samples were prepared as thin layers ($\geq 8 \mu\text{m}$) on a *p*-type boron-doped Si(100) wafer as described previously^{24,25}. Siloxene was prepared as described in ref. 11, and was stored in dry Ar immediately after preparation. Three types of siloxene specimens have been studied: freshly prepared, HF-treated and thermally annealed^{9,10} at 400 °C. X-ray absorption experiments were done on the double crystal InSb(111) monochromator beamline²⁶ at the Canadian Synchrotron Radiation Facility, Synchrotron Radiation Center, University of Wisconsin-Madison. Synchrotron-radiation-induced luminescence was recorded with a MacPherson 234M6 spectrometer which was equipped with a 0.2-m, 600-groove mm^{-1} spherical grating²⁴. Two types of optical measurements were made. In one, the optical luminescence spectrum in the

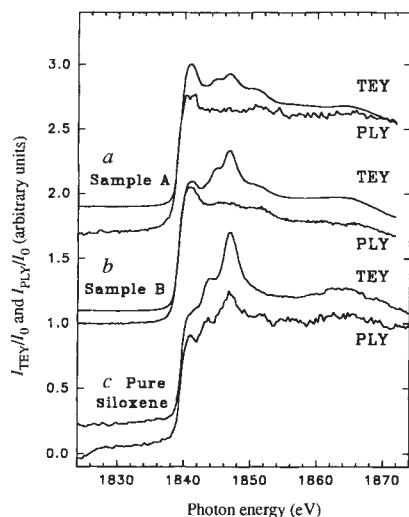


FIG. 2 Room-temperature spectra near Si K-edge of porous silicon produced at low current density (20 mA cm^{-2} for 20 min) and of siloxene. *a*, Porous silicon stored for two weeks (sample A); *b*, porous silicon stored for four weeks (sample B); *c*, pure siloxene. Spectra recorded with both TEY and PLY; spectra have been shifted vertically for clarity.

region of 200–900 nm was obtained at selected excitation energies below and above the absorption edge; in the other, the total luminescence yield (with the spectrometer set at zero order) was used to monitor the absorption as a function of X-ray energy. Total electron yield (TEY) measurements of XAFS were made concurrently with the photoluminescence yield (PLY) in a vacuum chamber. As Si has a large absorption cross-section above the K-edge (one absorption length is $\sim 1.3 \mu\text{m}$; ref. 27), nearly all the incident photons are absorbed by the relatively thick porous silicon layer on the Si substrate and by the siloxene sample. Under these conditions, TEY is sensitive to both near-surface and bulk Si atoms. PLY is a better probe of the bulk of the porous silicon; the photons have a significantly longer attenuation length ($5.9 \mu\text{m}$ for a typical wavelength of $\sim 740 \text{ nm}$, as estimated from the absorption constant of silicon in ref. 28) and the PLY is sensitive only to those absorption sites with a high quantum yield.

Figure 1 shows the TEY spectrum, taken near the Si K-edge, of a two-week-old, as-prepared porous silicon specimen, and the yield of a clean Si(100) sample. In the porous silicon spectrum, a peak, often referred to as a whiteline, occurs at the absorption edge jump ($\sim 1,840 \text{ eV}$) followed by a shoulder at $\sim 1,844 \text{ eV}$ and a relatively intense peak at $\sim 1,847 \text{ eV}$. The threshold (inflection point of the rising edge) has shifted slightly to higher energy ($\sim 0.35 \text{ eV}$) relative to crystalline Si, suggesting a wider band gap in porous silicon than in crystalline silicon^{14,15}. The peak at $\sim 1,847 \text{ eV}$ is a well-known feature of Si–O bonding in silicon oxide. The weak but clearly noticeable shoulder at $\sim 1,844 \text{ eV}$ is not a known feature of either silicon oxide or Si–O bonding in Si–methoxy compounds; it probably originates from hydride and hydroxide, as it also appears for siloxene (Fig. 2) and amorphous silicon (D.T.J., J. Du, T.K.S., B.Y. Tong and P.R. Norton, unpublished data). These three features are characteristic of all the as-prepared porous silicon samples, regardless of the current density used in the preparation and of ageing, although the relative intensities vary considerably. Finally, the characteristic sharp doublet in the whiteline of crystalline silicon coalesces into a broad single peak in the porous silicon spectrum (inset, Fig. 1). These observations have been taken as evidence for the quantum confinement model in two recent reports^{14,15}.

Figure 2 shows the TEY and PLY near-edge spectra of two typical porous silicon samples prepared at low current density (20 mA cm^{-2} for 20 min) and a freshly prepared siloxene. Samples A and B were stored under ambient conditions for two and four weeks, respectively, before the measurement. Several important features from Fig. 2 are noted. First, the TEY spectra of the two porous silicon samples are similar except for the relative intensity of the three-peak pattern above the threshold, where sample B shows a more intense oxide feature as expected. Second, both PLY spectra have the same features in the 1,844–1,850 eV region which differ from those observed in the TEY spectra; of particular interest is the disappearance of the Si oxide feature at 1,847 eV. Third, the PLY spectra beyond the whiteline are similar to the TEY spectrum of crystalline Si (Fig. 1). Assuming that the luminescence arises from the recombination of holes in the valence band with electrons in the conduction band, and that the creation of a core-hole and subsequent cascade in the absorbing atom helps this process locally, we can conclude that only Si sites that produce photoluminescence efficiently will contribute to the PLY spectrum. By comparing Fig. 2*a* and *b* it can be seen that the amount of silicon oxide on the sample surface (indicated by the size of the peak at $\sim 1,847 \text{ eV}$) has virtually no effect on the PLY spectra, so the surface silicon oxide is not responsible for the luminescence. Thus we can separate the XAFS of the Si sites that produce luminescence efficiently from those that do not. The PLY spectra of Fig. 2*a* and *b* therefore represent the near-edge structure of porous silicon sites that luminesce. This result confirms earlier observations that porous silicon is

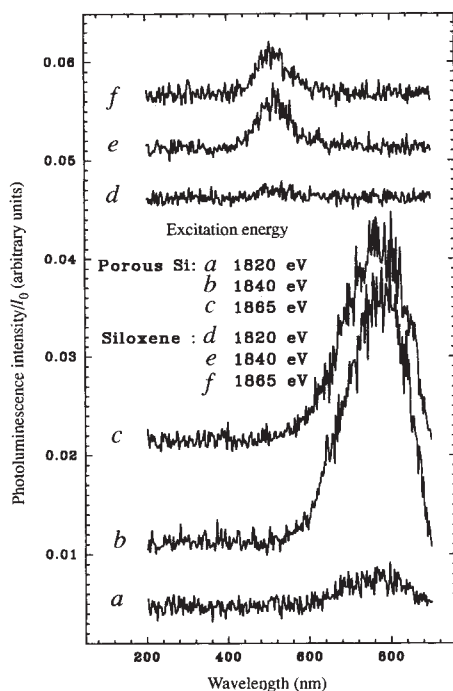


FIG. 3 Soft-X-ray-induced luminescence of porous silicon (sample A, Fig. 2) and siloxene (same sample as used to obtain Fig. 2c) at room temperature. Spectra have been shifted vertically for clarity.

essentially crystalline silicon^{6,14–16}. Finally, Fig. 2c shows the TEY and PLY near-edge spectra of a thin film of siloxene. The spectra are similar, but differ significantly from the PLY spectrum of porous silicon. These results show that the origin of luminescence in porous silicon is not the same as that of the

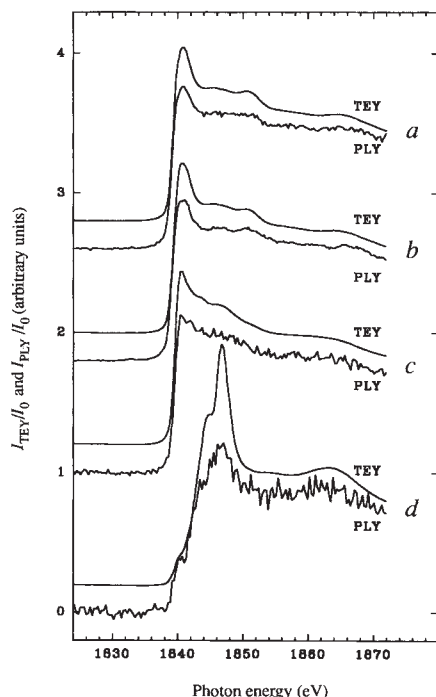


FIG. 4 Room-temperature spectra near Si K-edge of: a, porous silicon, sample A after treatment for 1 min in HF (48% aqueous); b, porous silicon, sample B after treatment for 5 s in HF (~15% aqueous); c, siloxene after treatment for 1 min in HF (48% aqueous); d, siloxene after annealing at 400 °C for 5 min in ambient atmosphere. Spectra have been shifted vertically for clarity.

freshly prepared siloxene. The three-peak pattern observed in Fig. 2c just above the threshold is attributed to the local chemical bonding of Si–Si, Si–H or Si–OH, and Si–O species, respectively. The details of the assignment will be discussed elsewhere.

Figure 3 shows the optical luminescence spectra of porous silicon and pure siloxene excited at several selected photon energies below and above the Si K-edge. The luminescence maximum for our siloxene sample appears at ~520 nm whereas the maximum for porous silicon appears at ~750 nm. These results are similar to those recorded with ultraviolet excitation. The maximum for luminescence induced by soft X-rays, for porous silicon varies considerably, in accord with the quantum confinement mechanism⁷: the higher the current density used in the preparation, the higher the porosity and the shorter the wavelength.

We next compare measurements of HF-treated porous silicon, HF-treated siloxene and thermally annealed siloxene. Treatment with HF before XAFS measurement removes the surface oxide. Figure 4a, b and c shows the TEY and PLY spectra of porous silicon samples A and B and siloxene after HF treatment; Fig. 4d shows the spectra for siloxene annealed at 400 °C in the ambient atmosphere. The spectra in Fig. 4a and b are essentially the same as the PLY spectra of the as-prepared porous silicon samples (Fig. 2) and closely resemble that of clean silicon (Fig. 1) beyond the whiteline, whereas in Fig. 4c we see that the HF-treated siloxene has very different features from porous silicon and pure siloxene. The corresponding luminescence spectra for the HF-treated porous silicon are similar to those for the untreated except that there is a small blueshift and the intensity increases, whereas the luminescence spectra for the HF-treated siloxene are less intense and their maxima have shifted to ~500 nm.

For the thermally oxidized siloxene (Fig. 4d), the corresponding optical luminescence maximum has shifted to 540 nm. The near-edge features are clearly very different from those of porous silicon. This observation is of special significance because it is the thermally annealed siloxene whose photoluminescence spectrum most closely resembles that of porous silicon^{9,10}.

From the above analyses we can conclude that porous silicon is essentially crystalline silicon locally, that the surface silicon oxide constituents are not responsible for the photoluminescence and, most important, that the luminescence is not of the same origin as that in pure siloxene, HF-treated siloxene or thermally annealed siloxene. Thus our results support the quantum confinement model. □

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- Pickering, C., Beale, M. I. J., Robertson, D. J., Pearson, P. J. & Greef, R. J. *J. Phys. C* **17**, 6535–6552 (1984).
- Canham, L. T. *Appl. Phys. Lett.* **57**, 1046–1048 (1990).
- Uhlir, A. *Bell Syst. Tech. J.* **35**, 333–347 (1956).
- Turner, D. R. *J. electrochem. Soc.* **105**, 402–408 (1958).
- Sailor, M. J. & Kavanagh, K. L. *Adv. Mater.* **4**, 432–434 (1992).
- Cullis, A. G. & Canham, L. T. *Nature* **353**, 335–338 (1991).
- Canham, L. T., Houlton, M. R., Leong, W. Y., Pickering, C. & Keen, J. M. *J. appl. Phys.* **70**, 422–431 (1991).
- Lehmann, V. & Gösele, U. *Appl. Phys. Lett.* **58**, 856–885 (1990).
- Déak, P., Rosenbauer, M., Stutzmann, M., Weber, J. & Brandt, M. S. *Phys. Rev. Lett.* **69**, 2531–2534 (1992).
- Brandt, S., Fuchs, H. D., Stutzmann, M., Weber, J. & Cardona, M. *Solid State Commun.* **81**, 307–312 (1992).
- Weiss, A., Beiland, G. & Mayer, H. Z. *Naturforsch.* **B34**, 25–30 (1979).
- Fathauer, R. W., George, T. & Ksendzov, A. *Appl. Phys. Lett.* **60**, 995–997 (1992).
- Vasquez, R. P., Fathauer, R. W., George, T., Ksendzov, A. & Lin, T. L. *Appl. Phys. Lett.* **60**, 1004–1006 (1992).
- van Buuren, T., Gao, Y., Tiedje, T., Dahn, J. R. & Way, B. M. *Appl. Phys. Lett.* **60**, 3013–3015 (1992).
- Sham, T. K. *et al. Can. J. Phys.* (in the press).
- Terry, J., Liu, H., Woicik, J., Cao, R. & Pianetta, P. *Vacuum Sci. Technol.* (In the press).
- Sham, T. K., Holroyd, R. A. & Munoz, R. *Nucl. Instrum. Meth. A* **249**, 530–535 (1986).
- Sham, T. K. *et al. Jpn J. appl. Phys.* **32**, suppl. 32–2, 223 (1993).
- Jpn J. appl. Phys.* **32**, suppl. 32–2 (1993).
- Bianconi, A., Jackson, D. & Monahan, K. *Phys. Rev.* **B17**, 2021–2024 (1978).
- Goulon, J., Tola, P., Lemonnier, M. & Dexpert-Ghys, J. *Chem. Phys.* **78**, 347–356 (1983).

22. Murata, T., Emura, S., Moriga, T., Maeda, H. & Normura, M. in *X-ray Absorption Fine Structures* (ed. Hasnain, S. S.) (Ellis Horwood, New York, 1991).
23. Emura, S. *et al. Phys. Rev. B* **47**, 6918–6930 (1993).
24. Sham, T. K. *et al. Proc. Symp. D Mat. Res. Soc. Mtg. Boston, November/December 1992* (eds Tu, C. W., Houghton, D. C. & Tung, R. W.) (MRS, Pittsburgh, in the press).
25. Coulthard, I., Lorimer, J. W. & Sham, T. K. *Abstr. 824RNP, 118th Mtg Electrochem. Soc. Toronto, October 1992*.
26. Yang, B. X. *et al. Nucl. Instrum. Methods Phys. Res. A* **316**, 422–436 (1992).
27. McMaster, W. H., Kerr Del Grande, N. & Hubbell, J. H. *Compilation of X-ray Cross-Sections* (National Technical Information Service, Springfield, VA, 1969).
28. *Handbook of Optical Constants of Solids* (ed. Palik, E. D.) (Academic, Orlando, 1985).

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Voltage-sensitive magnetic gels as magnetic resonance monitoring agents

Shachar Frank & Paul C. Lauterbur*

Biomedical Magnetic Resonance Laboratory, University of Illinois at Urbana-Champaign, 1307 West Park Street, Urbana, Illinois 61801, USA

MANY polymer gels undergo a volume phase transition in electric fields^{1–5}. We report here the synthesis of a polyelectrolyte gel that incorporates magnetic particles of iron oxide into the polymer network; these particles couple the gel volume transition to the NMR relaxation times of the surrounding water. We find that the water proton relaxation rates (T_2^{-1}) in aqueous suspensions of particles of the magnetic gel increase significantly in an electric field. Such changes can also be induced by the hyperpolarization of red blood cells in the suspension, presumably as a result of the electric fields at the cell membrane surfaces. These gels may play a part in magnetic resonance imaging analogous to that of voltage-sensitive dyes in optical imaging^{6–9}.

We used superparamagnetic iron oxide particles obtained from Polysciences, Warrington, Pennsylvania (# 19634). These were about 0.01–0.12 μm in diameter, and consisted of iron oxide crystals embedded in an equal weight of a polymer made from 95% styrene and 5% methyl methacrylate, with surface carboxylic acid groups. Allylglycine (2-amino-4-pentenoic acid) (Sigma, St. Louis, Missouri; #A8378) or oleylamine (Aldrich, Milwaukee, Wisconsin; #O-780-5) was covalently coupled to the particles by the carbodiimide method¹⁰. The particle suspension was dried under argon and the dry powder (~12 mg) suspended in 68.5 ml of cyclohexane (analytical grade) and 0.48 g of sorbitan monooleate (surfactant) in a round-bottomed flask. To this suspension we added 12.79 g sodium acrylate (Polysciences #01207, or synthesized from sodium methoxide (Aldrich #15,625-6) and acrylic acid (Aldrich #14,723-9)), which served as the gel monomer, along with 0.01 g *N,N*-methylene-bis-acrylamide (Sigma #M7229) (cross-linker) and 0.03 g potassium persulphate (initiator). The mixture was polymerized for 6 hours at 60 °C under an argon atmosphere, with constant stirring at 180–200 r.p.m. This procedure is similar to that described in ref. 11. The microparticles were then equilibrated with 100 ml of distilled water for 1 hour, washed once with 100 ml cyclohexane and four to six times with methanol, and dried in an argon gas stream. The dry precipitate (~0.05% iron oxide, w/w) was crushed with a mortar and pestle and suspended in 35 ml of distilled water. The resulting magnetic sodium polyacrylate gel microparticles were about 50–200 μm in diameter. They were uniformly brown in colour, indicating a uniform distribution of iron oxide particles in the gel. Portions of some batches of particles were homogenized and ultrasonicated to reduce the particle size to about 10–100 μm .

We observed that the gel microparticles underwent a volume phase transition in the presence of an applied electric field. We placed an aqueous suspension of microparticles dyed with methylene blue in an electrochemical cell constructed on a microscope slide, and applied a field of 40 V cm^{-1} . The particles shrank within a few minutes; larger particles shrank more slowly^{11–13}. The diameter of a typical particle decreased by about 36%, corresponding to a volume decrease of about 73%. The particles swelled more slowly back to their original size after the field was turned off.

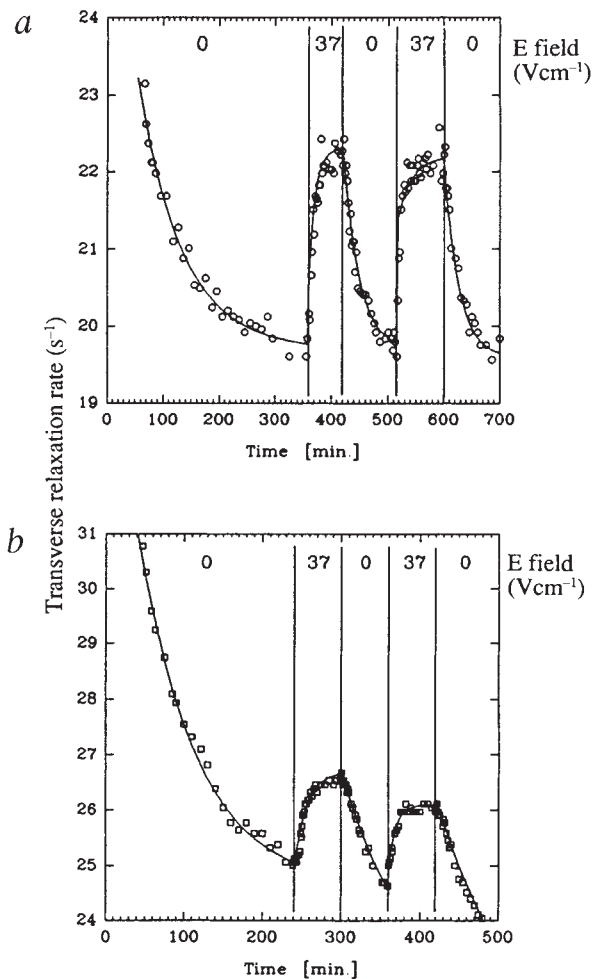


FIG. 1 Time course of the water proton transverse relaxation rates (T_2^{-1}), with and without an applied electric field. The lines are exponentials fitted independently to each segment. Suspensions of the magnetic polyelectrolyte gel microparticles were put in a rectangular glass cell. The inner sides of two opposite walls of the cell, separated by 2.80 mm, were coated in gold foil. In *a* the particles are superparamagnetic iron oxide-(allylglycine)-sodium polyacrylate (0.25mM Fe), and in *b* superparamagnetic iron oxide-(oleylamine)-sodium polyacrylate (0.5mM Fe). The cell was put inside an 8-cm internal diameter r.f. coil in a SISCO NMR spectroscopy imaging system (4.7 T, 33 cm bore). Cables were attached to the two gold electrodes in the cell and extended to a d.c. power supply. The NMR transverse relaxation time (T_2) of the sample was measured at room temperature using a Carr-Purcell-Meiboom-Gill (CPMG) phase-alternated spin-echo pulse sequence ($90^\circ_x - \tau - (-180^\circ_y - \tau - \text{echo})_n$) with a delay time τ of 0.003–0.008 s. Each T_2 was calculated from 15 echoes by fitting a single exponential. The iron-oxide particles alone were about an order of magnitude more effective in increasing T_2^{-1} than they were after incorporation into polyelectrolyte gels.

* To whom correspondence should be addressed.

We measured the changes to the water proton NMR transverse relaxation times (T_2) induced by the shrinking and swelling behaviour. Figure 1 shows two typical plots of transverse relaxation rates (T_2^{-1}) versus time for a field of 37 V cm^{-1} . This, like most samples, showed an initial decay of T_2^{-1} when inserted into the magnet, a phenomenon observed with other magnetic particles and attributed for those materials to aggregation caused by the external magnetic field^{14,15}. Application of an electric field causes the relaxation rates to increase. The average increase, for the first and second applications of an electric field, was $11.1 \pm 4.0\%$, including all 15 measurements made on five batches (not including the non-functioning batches described below), two with allylglycine and three with oleylamine. The relaxation rate drops back towards its earlier level when the electric field is turned off. Both the rise and subsequent fall are roughly exponential.

The electric-field-induced increase in T_2^{-1} occurs only with gels that undergo a volume phase transition. Batches prepared from an impure monomer that appeared to contain some free acrylic acid always produced gels that did not go through such a transition in the presence of an electric field, but were otherwise similar to materials that did. It seems probable, although not certain, that the impurity in the monomer was responsible for this anomalous behaviour. Although suspensions of particles

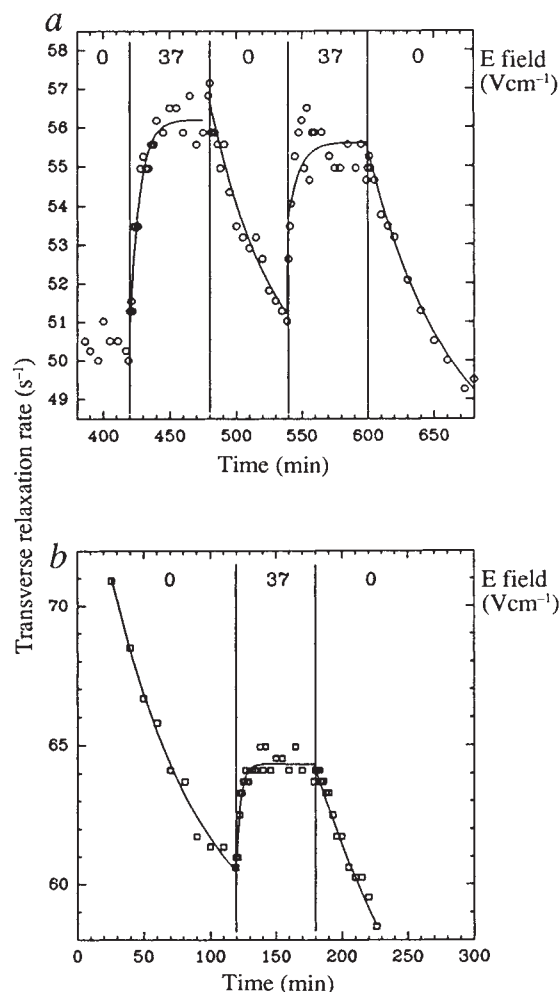


FIG. 2 Time course of the water proton transverse relaxation rates (T_2^{-1}), with and without an applied field, for suspensions of magnetic polyelectrolyte gel microparticles (superparamagnetic iron oxide-(oleylamine)-sodium polyacrylate) before (a, 0.86 mM Fe) and after (b, 0.80 mM Fe) reducing the particle size. The lines are exponentials fitted independently to each segment. The separation between the electrodes in the electrochemical cell was 3.1 mm (a) or 4.25 mm (b).

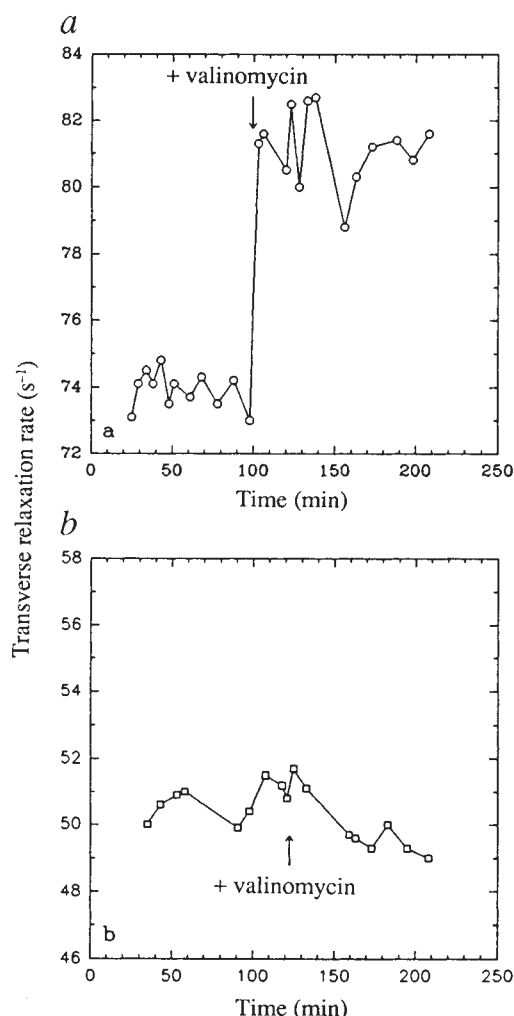


FIG. 3 Water proton transverse relaxation rates (T_2^{-1}) of a 10% hematocrit guinea-pig red blood cell suspension (146 mM NaCl, 4 mM KCl, 4 mM hepes buffer, 4 mM Tris buffer, pH 7.4) with 10% magnetic polyelectrolyte gel microparticles (0.24 mM Fe) before and after hyperpolarization of the cell membranes by adding valinomycin (0.1 μM final concentration) using (a) functioning and (b) non-functioning superparamagnetic iron oxide-(oleylamine)-sodium polyacrylate microparticles. Measurements were made at room temperature in a GN300/Libra 7.0 T NMR spectrometer using a CPMG pulse sequence with eight echoes and a delay time τ of 1.5 ms.

made from these gels usually showed an initial decrease in T_2^{-1} in a magnetic field, no increase in T_2^{-1} occurred when an electric field was applied. We conclude that the observed changes in T_2^{-1} with changes in the applied electric field are a result of the reversible volume phase transition of the magnetic gel microparticles, and not a result of their aggregation and disaggregation. This conclusion is strengthened by the result of an experiment in which the electric field had already been turned on when the gel suspension was inserted into the magnet. The usual slow decrease in relaxation rate was observed, followed by a much faster rate when the electric field was turned off. An even faster increase then occurred when the electric field was turned on again.

It was reported previously¹¹⁻¹³ that smaller polyelectrolyte gel microparticles shrink faster than larger ones. This was observed with the magnetic gel particles as well. Figure 2 shows the electric-field-induced changes of T_2^{-1} shown by two samples from one batch of particles. The average size of the particles in one sample was reduced by homogenization and ultrasonication. The rate of increase of the T_2^{-1} (using an exponential fit)

for the smaller particles is about 3 times as great as for the unprocessed sample with larger particles. Particles of the size obtained in our preparations are useful for some purposes (for example, studies of exposed surfaces of tissues and other objects, in coarsely porous materials and in cell preparations such as that described below), but many applications will require smaller, more rapidly responsive particles. Extrapolation of data obtained on similar but non-magnetic particles over a wide range of sizes suggests that particles of the order of 1 μm in size would contract in about 1 ms (refs 11–13). It has recently been found¹⁶ that a naturally occurring 5- μm polyelectrolyte granule has a voltage-induced swelling time of about 200 ms, in rough agreement with that predicted for a synthetic gel particle of the same size, as well as a faster (<30 ms) initial swelling time.

If magnetic gel microparticles were to go through a volume phase transition as a result of a change in the membrane potential of a nearby biological cell, they could be used as voltage-sensitive magnetic resonance monitoring agents in NMR imaging and spectroscopy, much as fluorescent dyes are being used for this purpose in light microscopy. NMR imaging might then be used in a new way to study electrical phenomena associated with cellular function, *in vitro* and *in vivo*.

To test this idea, we used the known effect of hyperpolarization of red blood cell membranes (from about -9 mV to about -66 mV) by valinomycin, an ionophore that makes the membrane selectively permeable to potassium¹⁷. Simple calculations¹⁸ suggested that the electric fields associated with the red blood cells would be much too short in range to affect the entire volume of a gel microparticle. But, rather to our surprise, addition of valinomycin (0.1 μM final concentration) to 10% by volume suspensions of guinea-pig red blood cells containing 10% (0.24 mM Fe) magnetic gel microparticles increased the water proton T_2^{-1} by $18 \pm 8\%$ in three experiments. An example is shown in Fig. 3. The non-functioning particles gave no effect. The maximum possible changes in ion concentrations in such systems are much too small to explain these results. It seems that the phenomena occurring in this cellular system are more complex than those that take place in a simple electrochemical cell.

Other polyelectrolyte gels are known to undergo volume phase transitions induced by changes in temperature, pH, the concentrations of various ions and molecules (such as saccharides) and redox reactions^{5,19–22}. We have shown here that they can also show sensitivity to cellular changes *in vitro*. Elucidating the mechanisms responsible and extending these studies to other cells and to tissues, organs and living organisms may give rise to a powerful new technique for studies of biological systems.

Magnetic species other than superparamagnetic particles may be combined with the gels. In two preliminary experiments, we found that the addition of paramagnetic Gd^{3+} (as GdCl_3) to the gel particles gave materials that showed a 5% and a 6% decrease in the water proton T_2^{-1} at 4.7 T on application of an electric field.

We can only speculate at present about the mechanisms that could account for these phenomena. Swelling and contraction may change the concentration of water molecules near the magnetic particles embedded in the gel and the diffusion coefficient and molecular rotational and translational correlation times of the water, as well as the direct magnetic interactions among the particles. These hypothetical mechanisms can be tested by more complex NMR experiments. They may all be involved in some degree, although one study²³ indicates that changes in the water diffusion coefficient may not be important. $\square$

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1. Tanaka, T., Nishio, I., Sun, S.-T. & Ueno-Nishio, S. *Science* **218**, 467–469 (1982).
2. Kwon, I. C., Bae, Y. H. & Kim, S. W. *Nature* **354**, 291–293 (1991).
3. Kajiwara, K. & Ross-Murphy, S. B. *Nature* **355**, 208–209 (1992).

4. Osada, Y., Okuzaki, H. & Hori, H. *Nature* **355**, 242–243 (1992).
5. Tanaka, J. *Polyelectrolyte Gels* (eds Harland, R. S. & Prud'homme, R. K.) 1–21 (ACS Symp. Series 480, Am. Chem. Soc., Washington, DC, 1992).
6. Salzberg, B. M., Davila, H. V. & Cohen, L. B. *Nature* **246**, 508–509 (1973).
7. Grinvald, A., Anglister, L., Freeman, J. A., Hildesheim, R. & Manker, A. *Nature* **308**, 848–850 (1984).
8. Cohen, L. *Nature* **331**, 112–113 (1988).
9. Kauer, J. S. *Nature* **331**, 166–168 (1988).
10. Daniel, J. C. *U.S. Patent No.* 4,064,080 (1977).
11. Kishi, R. & Osada, Y. *J. chem. Soc. Faraday Trans.* **185**, 655–662 (1989).
12. Hirose, Y., Amiya, T., Hirokawa, Y. & Tanaka, T. *Macromolecules* **20**, 1342–1344 (1987).
13. Matsuo, E. S. & Tanaka, T. *J. chem. Phys.* **89**, 1695–1703 (1988).
14. Ohgushi, M., Nagayama, K. & Wada, A. *J. magn. Reson.* **29**, 599–601 (1978).
15. Muller, R. N., Gillis, P., Moyny, F. & Roch, A. *Magn. Reson. Med.* **22**, 178–182 (1991).
16. Nanavati, C. & Fernandez, J. M. *Science* **259**, 963–965 (1993).
17. Rink, T. J. & Hladky, S. B. *Red Cell Membranes—A Methodological Approach* (eds Eliory, J. C. & Young, J. D.) 321–324 (Academic, New York, 1982).
18. Shaw, D. J. *Electrophoresis* (Academic, London, 1969).
19. Hirotsu, S., Hirokawa, Y. & Tanaka, T. *J. chem. Phys.* **87**, 1392–1395 (1987).
20. Osada, Y. *Adv. Polym. Sci.* **82**, 1–46 (1987).
21. Suzuki, A. & Tanaka, T. *Nature* **346**, 345–347 (1990).
22. Kokufuta, E., Zhang, Y.-Q. & Tanaka, T. *Nature* **351**, 302–304 (1991).
23. Corti, M., Pavesi, L., Rigamonti, A. & Tabak, F. *Phys. Rev.* **A43**, 6887–6893 (1991).

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Experimental evidence against CO_2 -promoted deep crustal melting

J. D. Clemens

Department of Geology, University of Manchester, Manchester M13 9PL, UK

THERE has been considerable controversy regarding the role of carbon dioxide in the metamorphism and melting of the Earth's crust, fuelled principally by reports of experimental evidence for CO_2 -enhanced partial melting of the assemblage phlogopite mica + quartz in the model system $\text{KAlO}_2\text{--MgO--SiO}_2\text{--H}_2\text{O--CO}_2^{1,2}$. The analogous process in the crust would lead to the formation of granulite-facies rocks at temperatures characteristic of amphibolite-facies metamorphism; the granulite facies^{3–5} would thus be indicative not of unusually high temperatures, but of the influx of carbonic fluids^{4,6}. Moreover, the similarity of their low-temperature melts to the compositions of some calc-alkaline lamprophyres led Peterson and Newton² to suggest that such magmas might be generated by the introduction of CO_2 -rich fluids, providing a means of large-scale mass transfer in the deep crust. These ideas have been highly influential⁶, but there are aspects of Peterson and Newton's results that are hard to reconcile with other available data. I have accordingly tried to replicate the results of refs 1 and 2 but, as I report here, have been unable to do so. The present results thus cast doubt on the general idea of melt fluxing by CO_2 , and the formation of magnesium- and CO_2 -rich magmas at crustal pressures.

The original basis for suspecting unusual behaviour in this system was the work of Wendlandt⁷, who reported relatively low-temperature melting when a mica-feldspar-quartz assemblage (a model for many crustal rocks) reacted with a $\text{CO}_2\text{--H}_2\text{O}$ fluid to produce a silicate melt (magma) and a pyroxene-bearing residuum (typical of granulite-facies metamorphic rocks). Wendlandt's results, at 800 MPa and 750 °C with $X^{\text{H}_2\text{O}}$ (mole fraction of H_2O in the fluid) = 0.5, do appear to show this reaction. However, his duplicate experiments, using starting materials with the same bulk composition but different crystalline phases, produced very different products. This suggests some form of metastability.

Peterson and Newton^{1,2} report melting, in the presence of $\text{CO}_2\text{--H}_2\text{O}$ fluids, at temperatures more than 50 °C lower than the modelled solidus at reduced activity of H_2O (refs 8,9), and CO_2 -enhanced melting at temperatures as much as 30 °C below

TABLE 1 Experimental run results

Run no.	Starting mix	Fluid (wt%)	Pressure (MPa)	Temperature (°C)	Time (h)	X ^F H ₂ O loaded	X ^F H ₂ O measured	Phase assemblage
260*	Phl+Qz	40	340	771	283	0.5	0.73	(Phl)+Qz+En+Gl+Fl
K-100	Phl+Qz	34	347.2±2.4	772±1	287	0.5	0.501	Phl+Qz
254*	Phl+Qz	50	340	785	112	0.5	0.38	(Phl)+Qz+En+Gl+(m)+Fl
K-104	Phl+Qz	49	342.0±1.4	784±1	112	0.5	0.409	Phl+Qz
K-105	En+Sa+Qz	51	342.0±1.4	784±1	112	0.5	0.301	(Phl)+Qz+En+Sa

Phl, phlogopite; Qz, quartz; En, orthoenstatite; Sa, high sanidine; m, "quench mica"^{1,2}; Gl, "glass"^{1,2}; Fl, supercritical fluid; (), minor amount of phase. 1 MPa = 10 bars pressure.

* Runs by Peterson and Newton^{1,2}.

the experimentally determined H₂O-saturated solidus. If correct, this would require that CO₂ should be more soluble in the melts than H₂O at the same pressure and temperature. However, experiments on a wide range of melt compositions (silicic to mafic and alkaline, silica-undersaturated) have shown that CO₂ solubility is 10 to 1,000 times lower than that of H₂O at the same experimental conditions¹⁰⁻¹⁵.

There are important disparities between the results of Peterson and Newton and the main body of other relevant experimental evidence. Their low-temperature melts are reported to coexist with quartz, yet the analytical data show them to be silica-undersaturated; they would seem to be well out of equilibrium with the solid phases present. The highly magnesian (~20 wt% MgO) character of these melts is also at odds with their low temperatures of formation. Silicate systems with higher MgO contents have higher melting temperatures.

Peterson and Newton² postulate that their very Mg-rich liquids could be metastable, because of kinetically inhibited growth of enstatite. However, pyroxenes nucleate and grow readily in experiments at high pressure and temperature, especially when fluid is present and in mafic compositions. Enstatite growth is a sensitive test for the beginning of melting reactions in this system¹⁶. Inhibited enstatite growth therefore seems an unlikely explanation for any metastability.

As the ideas put forward in the CO₂-fluxing theory are important, it seemed vital to verify the experimental results on which they are based. I used the same type of apparatus, an argon-pressurized, internally heated vessel, and platinum cap-

sules, with oxalic acid dihydrate to generate H₂O-CO₂ fluids with X^FH₂O = 0.5. The starting materials were finely ground mixtures of pure, stoichiometric, synthetic phlogopite, high sanidine, orthoenstatite and Brazilian optical-grade quartz, in proportions similar to those used by Peterson and Newton. In all respects, the experimental conditions were as similar as possible to those reported by Peterson and Newton for their runs 254 and 260. These two runs were chosen for duplication because they were reported to have produced melt (quenchable to a glass), at a temperature below that expected, with near-elimination of phlogopite and production of abundant enstatite. Also, the glass from run 254 had been analysed and is typical of their low-SiO₂, high-MgO material. Table 1 shows the results of the present work compared to those of Peterson and Newton^{1,2}. As in the previous work, values for final X^FH₂O were determined by freezing the capsules, then determining their sequential mass losses on puncture (to determine the mass of CO₂) and heating at 110 °C (for mass of H₂O). Uncertainties are large here and little significance should be attached to the numbers.

As is apparent from the table, attempts at duplication failed. Both sets of runs (K-100 and K-104/K-105) were clearly subsolidus, no reaction having taken place in either of K-100 or K-104 (Fig. 1a). Growth of phlogopite (verified by optical, X-ray diffraction and scanning electron microscope examinations) in run K-105 (Fig. 1b) confirms that phlogopite plus quartz is the stable assemblage at these conditions. Furthermore, detailed investigations of isobaric phase relations involving phlogopite, quartz, sanidine, enstatite, melt and

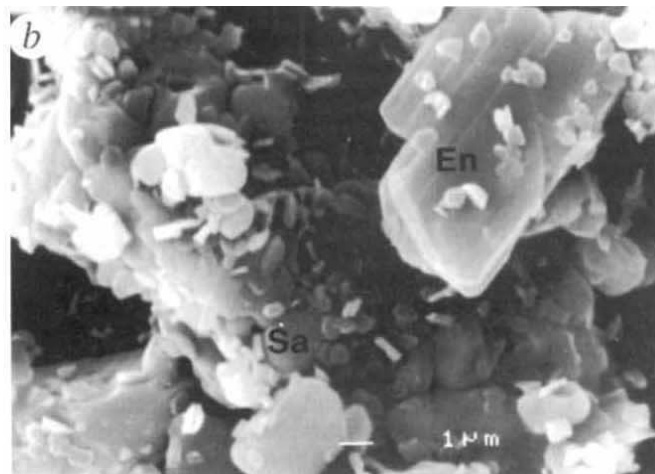
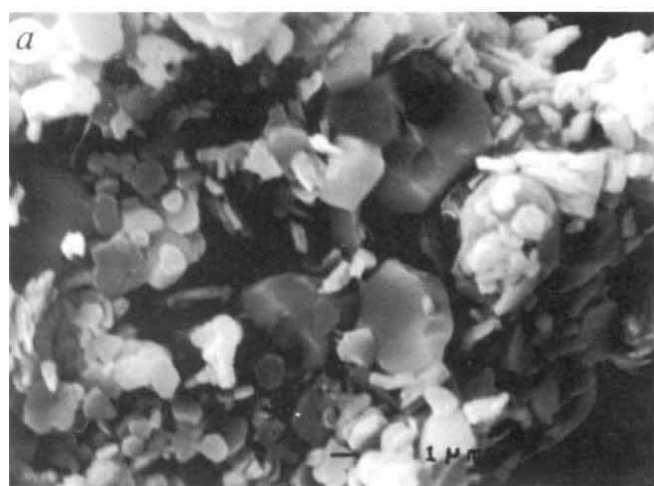


FIG. 1 Secondary electron images from scanning electron microscopy. a, Products of run K-104 showing unreacted quartz and phlogopite platelets. b, Run K-105 showing slightly corroded sanidine fragments

(Sa) and enstatite crystals (En) coated by spangles of newly formed phlogopite.

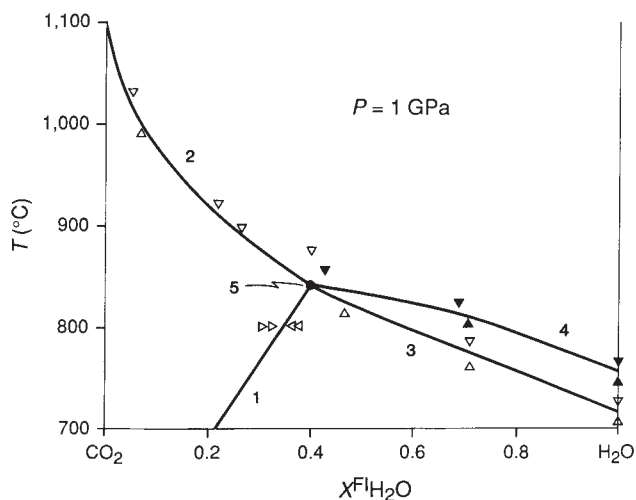


FIG. 2 Phase diagram of $X^{\text{FI}}\text{H}_2\text{O}$ against temperature T showing experimentally determined, quartz-saturated equilibria in the system $\text{KAlO}_2\text{--MgO--SiO}_2\text{--H}_2\text{O--CO}_2$ at 1 GPa. Reactions: (1). $\text{Phl} + \text{Qz} = \text{En} + \text{Sa} + \text{Fl}$; (2) $\text{En} + \text{Sa} + \text{Qz} + \text{Fl} = \text{M}$; (3) $\text{Phl} + \text{Sa} + \text{Qz} + \text{Fl} = \text{M}$; (4) $\text{Phl} + \text{Qz} + \text{Fl} = \text{En} + \text{M}$; (5) $\text{Phl} + \text{Qz} + \text{Fl} = \text{En} + \text{Sa} + \text{M}$, where M is hydrous silica-rich melt. Single open arrow heads bracket reactions 2 and 3; single filled arrow heads bracket reaction 4; double open arrow heads, reversal experiments on reaction 1.

TABLE 2 Examples of electron probe analyses of quench glasses

	Run K-93	Run K-71
$X^{\text{FI}}\text{H}_2\text{O}$	0.05	0.22
Coexisting phases	$\text{Qz} + (\text{En}) + \text{Gl}$	$\text{Qz} + (\text{En}) + \text{Gl}$
SiO_2	77.85 (0.69)	79.55 (0.88)
Al_2O_3	11.46 (0.42)	10.90 (0.51)
MgO	0.16 (0.04)	0.29 (0.2)
K_2O	10.53 (0.30)	9.24 (0.40)
Original probe total	93.71 (2.49)	94.77 (0.43)

Analyses are means of six points normalized to 100% anhydrous. Results are wt% oxide; numbers in parentheses are 1σ values. Counting losses on K_2O were negligible; samples were cooled to -197°C during analysis. Starting material was $\text{Qz} + \text{Sa} + \text{En}$; fluid was generated by loading $\text{Ag}_2\text{C}_2\text{O}_4 + \text{H}_2\text{O}$. A piston-cylinder apparatus was used, with platinum capsules containing the reactants. Pressure-transmitting media were talc-MgO (run K-93) or NaCl-MgO (K-71). Thermocouples were type R, Pt-Rh. Conditions for run K-93 were $1,002 \pm 16$ MPa, $1,025 \pm 1^\circ\text{C}$, 49 hours; for run K-71, 998 ± 28 MPa, $930 \pm 1^\circ\text{C}$, 9 hours.

$\text{CO}_2\text{--H}_2\text{O}$ fluids (J.D.C. and G. Droop, manuscript in preparation) reveal no thermal minima in the melting reactions at $X^{\text{FI}}\text{H}_2\text{O} < 1$, such as those postulated in Fig. 8 of Peterson and Newton². Figure 2 shows a summary of these new results at a pressure of 1 GPa. As can be seen, fluid-present melting reactions all consistently shift toward higher temperatures as $X^{\text{FI}}\text{H}_2\text{O}$ is lowered (to more CO_2 -rich compositions). All melt compositions, along the solidus at $X^{\text{FI}}\text{H}_2\text{O}$ between 0.05 and 1.0, are siliceous and low in MgO, with no quench crystals present (see Table 2 for examples). These results, and the preceding discussion, support the following conclusions.

First, CO_2 has no melt-fluxing effect in systems such as the one investigated here. On the contrary, as many experiments

have shown, it inhibits fusion in H_2O -bearing systems^{17–22}. Crustal melting in the presence of $\text{H}_2\text{O--CO}_2$ mixed fluids will always occur at higher temperatures than with pure H_2O fluid. Second, magmas produced by such melting will be granitic, with relatively high SiO_2 and low MgO contents, irrespective of $\text{H}_2\text{O--CO}_2$ ratio in any coexisting fluid phase. They will not contain appreciable dissolved CO_2 . Third, there is sound geological evidence that the passage of hot CO_2 can cause local dehydration. Classic examples are the incipient 'charnockites' (pyroxene-quartz-feldspar rocks) produced along fractures in biotite-bearing tonalitic gneisses in southern India^{23,24}. However, CO_2 is unlikely to be a significant agent in promoting regional granulite-grade metamorphism, melting, magma generation, metasomatism or long-range silicate mass transfer in the crust.

At present, there seems to be no satisfactory explanation for the aberrant results reported by Peterson and Newton. Suggested reasons for possible metastable behaviour^{1,2} are unconvincing. It is possible that the rounded globular bodies, termed 'glass' and interpreted as quenched melt, are indeed amorphous fluid-phase quench products^{1,2}. The presence of highly birefringent margins to these² may indicate association with fine secondary phlogopite. If this fine mica was included in bulk analysis of the sample, it might explain the high MgO contents found by Peterson and Newton. My results were acquired using effectively identical starting materials and experimental procedures, yet failed to reproduce the low-temperature melting phenomena or fluid-phase quench products. The fact that the same phase assemblage (phlogopite plus quartz) has been produced using two different starting materials suggests that the present experiments closely approach equilibrium. It is therefore difficult to ascribe the disparate observations to variations in sample treatment or to the intrinsic behaviour of the sample in this system. □

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- Peterson, J. W. & Newton, R. C. *Nature* **340**, 378–380 (1989).
- Peterson, S. W. & Newton, R. C. *Am. Miner.* **75**, 1029–1042 (1990).
- Newton, R. C., Smith, J. V. & Windley, B. F. *Nature* **288**, 45–50 (1980).
- Newton, R. C. in *Fluid–Rock Interactions During Metamorphism, Advances in Physical Geochemistry*, Vol 5 (eds Walther, J. V. & Wood, B. J.) 36–59 (Springer, New York, 1986).
- Touret, J. L. R. *C. R. Acad. Sci. Paris* **D271**, 2228–2231 (1970).
- Rogers, J. J. W., Satterfield, M. E., Skjerlie, K. P. & Johnston, A. D. *Geology* **21**, 89–90 (1993).
- Wendlandt, R. F. *Am. Miner.* **66**, 1164–1174 (1981).
- Burnham, C. W. *Phys. Chem. Earth* **13**, 197–229 (1981).
- Burnham, C. W. & Nekvasil, H. *Am. Miner.* **71**, 239–263 (1986).
- Blank, J. G., Stolper, E. M., Sheng, J. & Epstein, S. *Geol. Soc. Am. Abstr. Prog.* **21**, A157 (1989).
- Fogel, R. A. & Rutherford, M. J. *Am. Miner.* **75**, 1311–1326 (1990).
- Pan, V., Holloway, J. R. & Hervig, R. L. *Geochim. cosmochim. Acta* **55**, 1587–1595 (1991).

- Pawley, A. R., Holloway, J. R. & McMillan, P. F. *Earth planet. Sci. Lett.* **110**, 213–225 (1992).
- Taylor, W. R. *Eur. J. Miner.* **2**, 547–563 (1990).
- McMillan, P. F. & Holloway, J. R. *Contrib. Miner. Petrol.* **97**, 320–332 (1987).
- Vielzeuf, D. & Clemens, J. D. *Am. Miner.* **77**, 1206–1222 (1992).
- Ebadi, A. & Johannes, W. *Contrib. Miner. Petrol.* **106**, 286–295 (1991).
- Eggler, D. H. & Kadik, A. A. *Am. Miner.* **64**, 1036–1048 (1979).
- Keppler, H. *Contrib. Miner. Petrol.* **102**, 321–327 (1989).
- Millhollen, G. L., Wyllie, P. J. & Burnham, C. W. *Am. J. Sci.* **271**, 473–480 (1971).
- Novgorodov, P. G. & Shkodzinskiy, V. S. *Geochem. int.* **11**, 522–531 (1974).
- Wyllie, P. J. & Tuttle, O. F. *Am. Miner.* **65**, 411–437 (1959).
- Harris, N. B. W. & Bickle, M. J. *Earth planet. Sci. Lett.* **93**, 151–156 (1989).
- Stähle, H. J., Raith, M., Hoernes, S. & Delfs, A. J. *Petrol.* **28**, 803–834 (1987).

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Deep seismic profile across a Proterozoic collision zone: surprises at depth

S. B. Lucas*, A. Green†, Z. Hajnal‡, D. White*, J. Lewry§, K. Ashton||, W. Weber¶ & R. Clowes#

* Geological Survey of Canada, 601 Booth Street, Ottawa, Ontario, Canada, K1A 0E8

† Institut für Geophysik, ETH-Hönggerberg, CH-8093, Zurich, Switzerland

‡ Department of Geological Sciences, University of Saskatchewan, Saskatoon, Saskatchewan, Canada, S7N 0W0

§ Department of Geology, University of Regina, Regina, Saskatchewan, Canada, S4S 0A2

|| Saskatchewan Geological Survey, 1914 Hamilton Street, Regina, Saskatchewan, S4P 4V4

¶ Manitoba Geological Surveys Branch, 535-330 Graham Avenue, Winnipeg, Manitoba, Canada, R3C 4E3

Lithoprobe, 357 Geological Sciences Centre, 6339 Stores Road, The University of British Columbia, Vancouver, British Columbia, Canada, V6T 1Z4

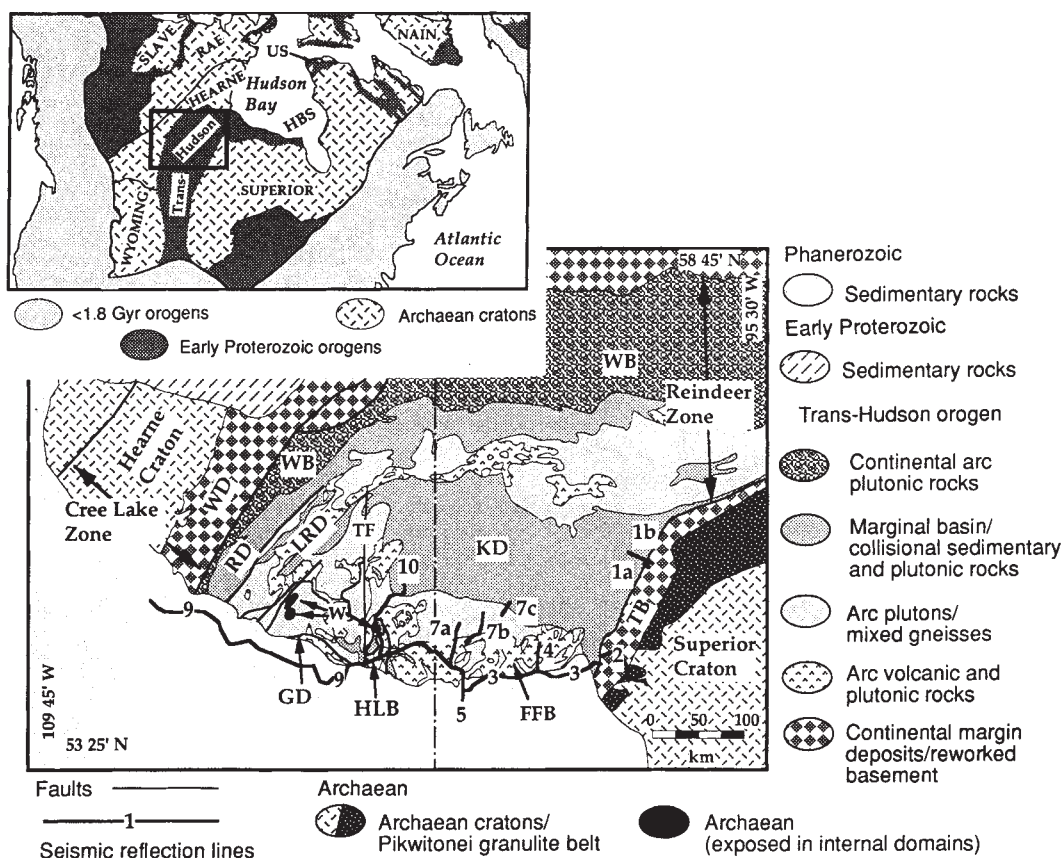
COLLISIONS between continents generate the world's most striking mountains; the deep structure of these collision zones, inferred from seismic reflection profiles¹⁻³, can be equally dramatic, if less well understood. Improved knowledge of the structure of ancient collisional belts, now known to have developed through plate tectonic processes^{4,5}, can provide important new insights into the mechanisms of crustal growth. Although many such belts have been partly destroyed by severe post-collisional deformation^{6,7} or magmatism⁸, some have been preserved in the stable interiors of continents⁹: one example is the Early Proterozoic Trans-Hudson orogen^{9,10}, in western Canada.

Here we present seismic reflection images across the entire orogen, which reveal a broadly symmetric structure, with reflections linked to island-arc rocks dipping beneath both bounding Archaean cratons. The observed reflection geometries imply a doubling of crustal thickness during the collision, along crust-penetrating faults. The presence of a significant amount of Archaean crust at depth, the extent of which could not have been determined from surface mapping, suggests the need for caution in inferring crustal growth rate from the surface area of juvenile crust^{11,12}.

The Trans-Hudson orogen (THO) forms part of a worldwide network of Proterozoic orogens that weld together Archaean provinces, and formed the connective tissue of a Proterozoic supercontinent^{9,13}. In the study area, the THO is distinguished by the largest exposure of juvenile, arc-derived Proterozoic crust in the Canadian Shield and is bounded by the Archaean Superior and Hearne cratons (Fig. 1)^{10,14}. More than 1,000 km of vertical-incidence seismic reflection data were obtained on and directly adjacent to the Canadian Shield (Fig. 1) as part of a LITHOPROBE¹⁵ programme in THO. The east-west corridor (bold lines 2, 3, 9 on Fig. 1) extended from the Superior craton in the east to the Hearne craton in the west and thus traversed the entire continent-continent collision zone.

The regions affected by Proterozoic orogeny comprise four principal tectonic domains^{10,16} (Fig. 1): (1) a narrow eastern foreland (Thompson belt)¹⁷; (2) a broad collage of imbricated magmatic arc, marginal basin and collisional basin rock (Reindeer zone) which structurally overlies Archaean material exposed in three small basement windows¹⁸ ('W' on Fig. 1); (3) an Andean-type continental margin batholith (Wathaman-Chipewyan batholith)¹⁹; and (4) a broad, reworked northwestern hinterland (Cree Lake zone)¹⁰. Data from the Hudson Bay and Ungava segments of the THO, northeast of the study area (HBS, US; Fig. 1 inset), suggest that continental rifting and oceanic spreading was initiated about 2.1–2.0 Gyr ago²⁰. Within the study area, convergent margin arc magmatism and arc

FIG. 1 Inset map shows the location of the study area with respect to the Trans-Hudson and other >1.8-Gyr-old Proterozoic orogens (dark grey) and Archaean provinces (named) of continental North America, Greenland and western Scandinavia (after ref. 9). All younger orogens are shaded light grey and autochthonous platform cover has been removed. HBS, US: Hudson Bay and Ungava segments of the Trans-Hudson orogen. Main map: locations of the seismic reflection lines (numbered) in relation to the geology. 'W' indicates location of Archaean basement windows within the Reindeer zone. FFB: Flin Flon belt; GD: Glennie domain; HLB: Hanson Lake block; LRD: La Ronge domain; KD: Kisseynew domain; RD: Rottenstone domain; TB: Thompson belt; TF: Tabbarnor fault; WB: Wathaman-Chipewyan batholith; WD: Wollaston domain.



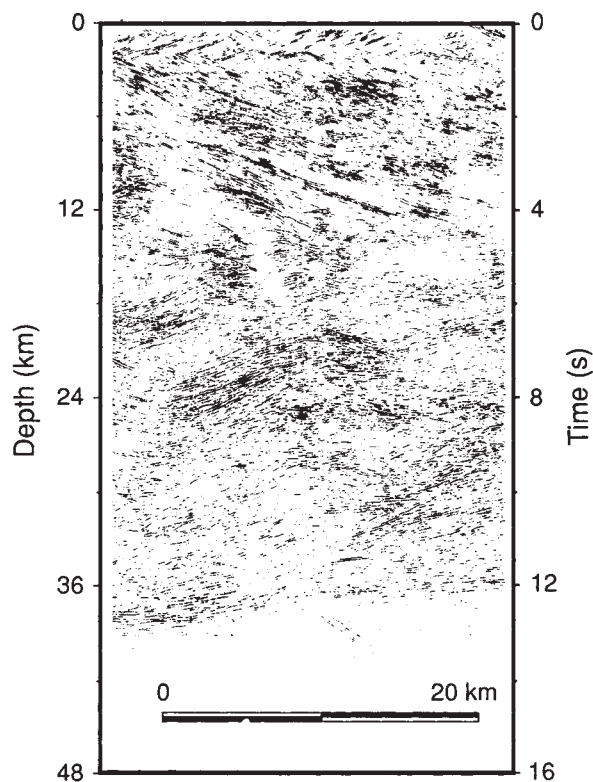


FIG. 2 Migrated seismic reflection profile showing a part of line 9 at true scale, assuming a constant migration velocity of 6.0 km s^{-1} . These data were acquired using a 240-channel telemetry acquisition system, with 50 m geophone group spacing, 100 m source group spacing and four vibrator trucks sweeping eight times at each shot station and using a 14 s linear upsweep from 12 to 56 Hz. The processing sequence applied to these data to obtain the migrated section included crooked-line binning, deconvolution, time-variant bandpass filtering, weathering static corrections, detailed velocity analysis, residual and trim static corrections, and random noise attenuation. Constant velocity (6.0 km s^{-1}) migration was applied for all lines except 1–3; for these lines, dip-moveout (DMO) corrections were applied and 90% DMO velocities were used for subsequent f-k migration.

accretion occurred between 1,910 and 1,830 Myr ago^{12,21}. Emplacement of the ~1,855-Myr-old Wathaman–Chipewyan batholith¹⁹ marked the onset of accretion to the Hearne cratonic margin, broadly coeval with widespread plutonism and initial molasse sedimentation throughout the Reindeer zone^{12,21}. Terminal collision of accreted arcs and Hearne craton with the Superior craton occurred between 1,830 and 1,790 Myr ago¹². Post-collisional deformation (~1,790–1,720 Myr ago²²) included movement along important orogen-parallel faults¹⁷ (TF, TB; Fig. 1) and continued shortening across the orogen.

High-quality seismic data reveal reflective Proterozoic and Archean crust from near surface to the crust–mantle boundary (examples are shown in Figs 2, 3a and 3b). Strong, east-dipping reflections dominate in the eastern two thirds of the orogen (line 3, Fig. 3b) and uniformly west-dipping events prevail in the west (west part of line 9, Fig. 3a). The marked dip reversal defines a crustal-scale culmination about which the orogen is crudely symmetrical; a similar structure was imaged on a recent seismic survey of THO near the border between Canada and the United States²³. It is noteworthy that reflections dip beneath the Archean margins on both sides of the orogen ('A', Fig. 3a and b), apparently contradicting the generally accepted tectonic model that Archean crust of the Superior craton was subducted beneath the orogen to the northwest^{14,16,17}. The well defined reflection Moho ('M' at 12–15 s on line 9) has been traced for a

distance >500 km as either a distinct zone of continuous reflections separating reflective crust from significantly less reflective mantle, or an abrupt decrease in reflectivity with depth. The lowermost 2–3 s of the crust is marked by dominantly subhorizontal events which appear to truncate overlying dipping reflections.

A geological interpretation of the composite reflection profile reveals several fundamental features of THO (Fig. 3c). Reflections 'A' (Fig. 3a and b) dipping beneath the bounding Archean cratons project updip to exposed Proterozoic magmatic arc (LRD, FFB; Fig. 3c) and marginal and collisional basin rocks (KD). Although this is consistent with the documented continental arc on the western side of the orogen^{16,19}, there is no evidence for an arc built on the Superior craton margin (Fig. 1)¹⁴. Efforts to reconcile the east-dipping reflections with the previously proposed models for west-dipping subduction of the Superior margin^{14,16,17} lead to the conclusion that the imaged structures along the eastern margin of the orogen were generated during terminal collision and/or subsequent intracontinental deformation. Reworked Archean crust in the Thompson belt (TB, Fig. 3b and c) is marked by east-dipping reflections whereas the preserved Archean Pikwitonei granulites to the east (PGB) are marked by west-dipping events. In our interpretation (Fig. 3c), the Pikwitonei granulites are structurally floored by a gently east-dipping fault coincident with the top of the east-dipping reflections imaged at about 5 s (16 km) at the east end of line 2 (see arrow, Fig. 3b). Such geometry, coupled with west-vergent (that is, east-dipping) shear zones along the Thompson belt^{17,24}, suggests that the collision of the accreted terranes of the Reindeer zone with the Superior cratonic margin occurred in such a way as to cause delamination of the Superior lithosphere near the base of the crust. This model proposes that the eastward-tapering Reindeer zone wedge underthrust the Superior crust and overthrust its lithospheric mantle in a manner analogous to the delamination of the European plate by the Apulian indenter³.

Three main lithotectonic packages are present in the uniformly east-dipping stack imaged on line 3 (Fig. 3b and c). High-grade arc-derived gneisses (highly reflective; NGC, Fig. 3b) underlie low-grade arc volcanic and plutonic rocks (scattered, diffuse energy; FFB, Fig. 3b), which in turn are juxtaposed to the east against medium/high-grade marginal and collisional basin sedimentary rocks whose grade and dip increase eastward (KD, Fig. 3b). Some strong upper-crustal reflections display curvilinear geometries reminiscent of flat-ramp-flat thrust fault geometries (for example, 'L' and 'D' in Fig. 3b), but the most prominent reflection packages have moderate dips from near-surface into the middle and lower crust ('R'). Data from the cross-profiles indicate that line 3 is roughly a true dip line; the implied thickness of the composite crustal section (~100 km) suggests substantial thickening associated with development of the accretionary collage during collisional tectonics. The overall geometry indicates crustal-scale imbrication, with thick sheets of mid/lower crustal rock stacked below a major detachment carrying the upper crustal remnants of the island arc complex. Two qualifications accompany this interpretation. The three-dimensional picture offered by the cross-profiles indicates that the collision was highly oblique to the Superior cratonic margin (see also refs 9, 14, 17), and structures possibly associated with extensional tectonics^{6,25,26} have been identified on the profiles: subhorizontal reflections at the base of the crust ('M', Fig. 3a), a core-complex-like antiform (culmination on line 9, Fig. 3a and b) and at least one major crust-penetrating detachment juxtaposing low-grade on higher-grade rocks ('DT', Fig. 3b).

The culmination extending throughout the crust in the central part of line 9 (Fig. 3a) is along strike from a well documented regional antiform that contains two of the structural windows in which Archean rocks are exposed¹⁸ ('W', Fig. 1). This observation, coupled with Sm–Nd isotopic analyses of drillcore samples

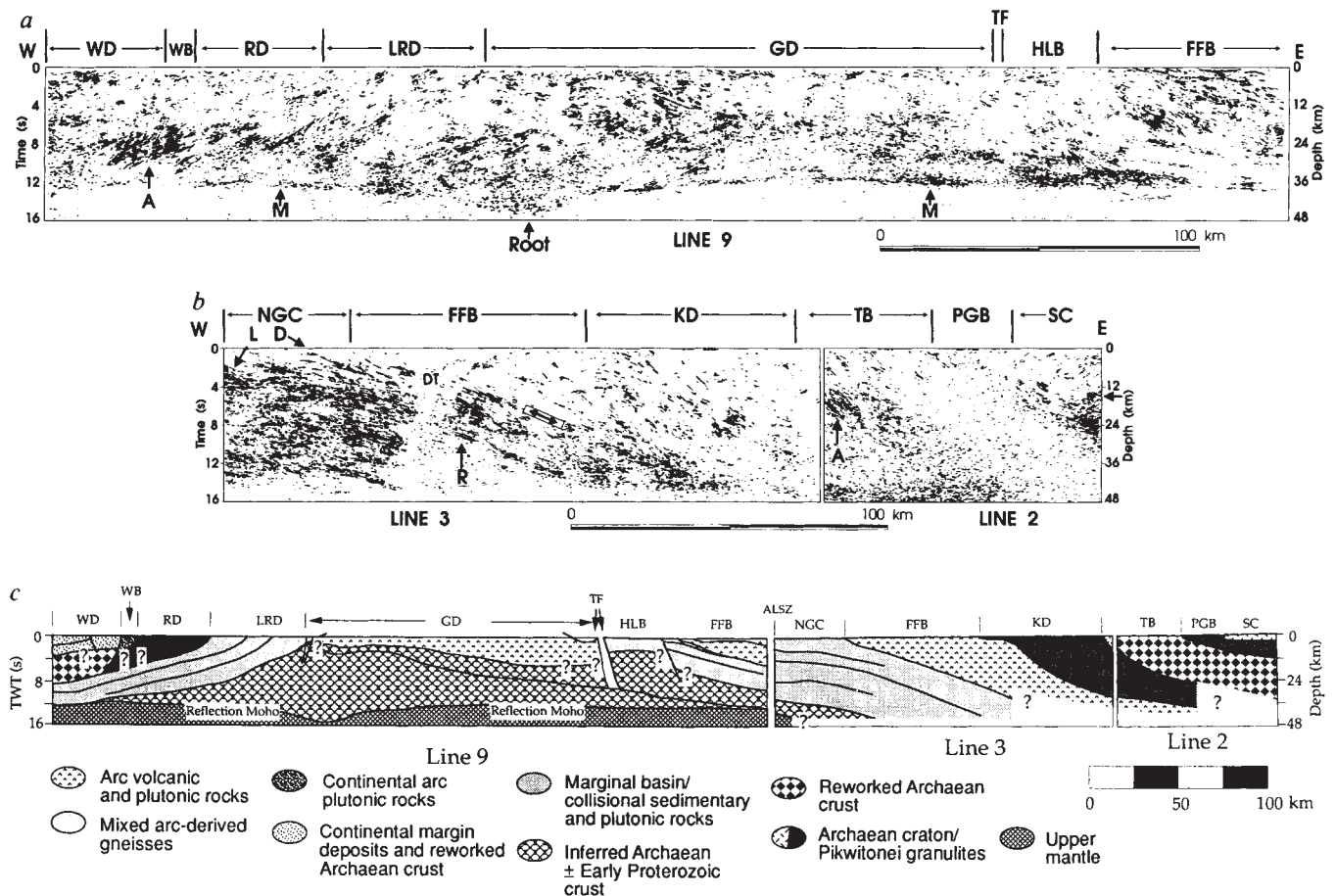


FIG. 3a Line drawing of highly coherency-filtered, migrated data derived using an automated routine showing main reflections on the western half of the THO composite profile (line 9), and shown at true scale assuming a constant migration velocity of 6.0 km s^{-1} . The principal features of line 9 are the reflections 'A' dipping under the bounding Archaean craton (WD: Wollaston domain), the well defined reflection Moho ('M'), and the reversal in the dip of reflections, centred on the crustal root ('Root'), which defines a culmination at the scale of the entire crust. Abbreviations as in Fig. 1. b, As a, but for main reflections on the eastern half of the THO composite profile (lines 2 and 3). The principal features of lines 2 and 3 are laterally coherent reflections similar to flat-ramp-flat thrust fault geometries ('L', 'D'), reflection packages extending linearly from near-surface into the middle and lower crust ('R'), a detachment separating highly reflective crust from overlying crust marked by scattered and diffuse energy ('DT'), and reflections 'A' dipping under the bounding Archaean craton (TB: Thompson belt, PGB: Pikwitonei granulite belt, SC: Superior craton). Arrow along the right side of line 2 shows the east-dipping boundary that separates a domain of heterogeneous, dominantly

west-dipping reflections above from uniformly east-dipping reflections below. NGC: Nameless Gneiss complex. c, Interpretive geological section for the composite reflection profile shown in a and b, derived from the integration of surface and drillcore geology with potential field maps and the seismic reflection profiles. The east end of line 9 is offset $\sim 50 \text{ km}$ to the northwest of the west end of line 3, and west end of line 2 is offset $\sim 12 \text{ km}$ to the north of the east end of line 3. Data were acquired along line 5 (Fig. 1) to connect lines 3 and 9. A late fault (ALSZ: Athapuskow Lake shear zone²⁹) occurs between the west end of line 3 and the east end of line 9 along line 5, and effects on apparent west-side-down (line 9) displacement in the plane of the composite profile. The culmination defined in line 9 by a reversal in reflection dip extending throughout the entire crust is interpreted to be cored by Archaean material possibly interleaved with and/or intruded by Proterozoic rocks. Thick-skinned stacking of arc (FFB, NGC³⁰) and basinal (KD) units on line 3 is suggested by the presence of reflection packages ('R', Fig. 3b) extending throughout the crust in the moderately east-dipping homocline.

of plutonic rocks to the south that yield mid-to-late-Archaean model ages²⁷, suggests that the culmination may largely consist of Archaean crust in its core (Fig. 3c). The interpretation is consistent with structural models of stacking of arc and marginal basin allochthons above a regional sole thrust with Archaean basement in its footwall^{14,16}. The proposed Archaean crust in the core of the antiformal is structurally isolated from the Archaean cratons bounding the orogen and may thus be exotic (allochthonous). U-Pb and Sm-Nd isotopic data from the Reindeer zone indicate that 1,910–1,830-Myr magmatic rocks are largely juvenile whereas post-1,790-Myr pegmatites yield Archaean Nd model ages, suggesting that underthrusting of the Archaean material beneath the Reindeer zone occurred between 1,830 and 1,790 Myr ago¹², the inferred age of terminal collision between the Reindeer zone and the Superior craton²². It is noteworthy that the culmination on line 9 coincides with a

relatively narrow (50 km) crustal root indicated by an increase in depth to reflection Moho from 12 to 15 s ('Root', Fig. 3a). The root is characterized by shallow-dipping, concave-up reflections that are contiguous with subhorizontal events defining the base of the crust to the east and west (Fig. 3a). The ratio of Archaean to Proterozoic material within the core of the culmination, the origin of this allochthonous block and the process(es) generating the crustal-scale culmination, its internal structures and its associated root remain enigmatic.

The reflection images map structures generated at various intervals in a complex tectonic history that included crustal-scale overthrusting, terminal collision with delamination of the Superior cratonic margin and underthrusting of an 'exotic' Archaean block, and subsequent intracontinental deformation. The gross crustal architecture of the orogen was largely shaped during this collisional and intracontinental deformation, which

had the cumulative effect of destroying most older, subduction-related structures. Inferences of subduction polarity in other ancient orogens drawn from either reflection geometry or surface structures therefore need to be critically reviewed. A fundamental characteristic is the predominance of structures extending throughout the crust (for example, detachment 'DT', Fig. 3b; Archæan-cored culmination, Fig. 3c), suggesting that collisional and/or intracontinental deformation was principally 'thick-skinned'^{1,20,28}. Clearly, interpretations of crustal structure and tectonic history in collisional orogens based solely on surface geology can be flawed. Furthermore, crustal growth rate¹¹ based on the surface area of juvenile crust preserved in orogens such as THO need to be reconsidered¹². □

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- Green, A. G. *et al.* *Geology* **16**, 788–792 (1988).
- Meissner, R. *et al.* (eds) *Am. geophys. Un. Geodynam. Series* **22**, 1–450 (1991).
- Frei, W. *et al.* *Nature* **340**, 544–548 (1989).
- Klemperer, S. *et al.* *Nature* **348**, 34–38 (1990).
- Scott, D. J., Helmstaedt, H. & Bickle, M. J. *Geology* **20**, 173–176 (1992).
- McCarthy, J. & Thompson, G. A. *Geol. Soc. Am. Bull.* **100**, 1361–1374 (1988).
- Keen, C., Peddy, C., de Voogd, B. & Matthews, D. *Geology* **17**, 173–176 (1989).
- Nelson, K. D. *Geophys. J. int.* **105**, 25–35 (1991).
- Hoffman, P. F. in *The Geology of North America — An Overview* (eds Bally, A. W. & Palmer, A. R.) 447–512 (Geol. Soc. Am., Boulder, 1989).
- Lewry, J. F. & Collerson, K. D. in *The Early Proterozoic Trans-Hudson Orogen of North America* (eds Lewry, J. F. & Stauffer, M. R.) 1–14 (Geol. Assoc. Can. spec. Pap. 37, 1990).

- Patchett, P. J. & Arndt, N. T. *Earth planet. Sci. Lett.* **78**, 329–338 (1986).
- Bickford, M. E., Collerson, K. D., Lewry, J. F., Van Schmus, W. R. & Chiarenzelli, J. R. *Geology* **18**, 14–18 (1990).
- Hoffman, P. F. *Geology* **17**, 135–138 (1989).
- Green, A. G., Hajnal, Z. & Weber, W. *Tectonophysics* **116**, 281–322 (1985).
- Clowes, R. M. *et al.* *Can. J. Earth Sci.* **29**, 1813–1864 (1992).
- Lewry, J. F. *Nature* **294**, 69–72 (1981).
- Bleeker, W. in *The Early Proterozoic Trans-Hudson Orogen of North America* (eds Lewry, J. F. & Stauffer, M. R.) 57–73 (Geol. Assoc. Can. spec. Pap. 37, 1990).
- Lewry, J. F., Thomas, D. J., Macdonald, R. & Chiarenzelli, J. in *The Early Proterozoic Trans-Hudson Orogen of North America* (eds Lewry, J. F. & Stauffer, M. R.) 75–94 (Geol. Assoc. Can. spec. Pap. 37, 1990).
- Meyers, M. T., Bickford, M. E. & Lewry, J. F. *Geol. Soc. Am. Bull.* **104**, 1073–1085 (1992).
- St-Onge, M. R., Lucas, S. B. & Parrish, R. *Can. J. Earth Sci.* **29**, 746–764 (1992).
- Gordon, T. M., Hunt, P. A., Bailes, A. H. & Syme, E. C. in *The Early Proterozoic Trans-Hudson Orogen of North America* (eds Lewry, J. F. & Stauffer, M. R.) 177–199 (Geol. Assoc. Can. spec. Pap. 37, 1990).
- Machado, N. in *The Early Proterozoic Trans-Hudson Orogen of North America* (eds Lewry, J. F. & Stauffer, M. R.) 433–441 (Geol. Assoc. Can. spec. Pap. 37, 1990).
- Nelson, K. D. *et al.* *Geology* (in the press).
- Fuente, F. & Robin, P.-Y. *Can. J. Earth Sci.* **26**, 1976–1989 (1989).
- Goodwin, E. B. & Thompson, G. A. *Geol. Soc. Am. Bull.* **100**, 1616–1626 (1988).
- Cook, F. A. *et al.* *Tectonics* **11**, 12–36 (1992).
- Collerson, K. D., Lewry, J. F., Bickford, M. E. & Van Schmus, W. R. in *Modern Exploration Techniques*, 150–165 (Saskatchewan Geol. Soc., Regina, 1991).
- Goleby, B. R., Shaw, R. D., Wright, C., Kennett, B. L. N. & Lambeck, K. *Nature* **337**, 325–330 (1989).
- Syme, E. C. in *Report of Field Activities*, 20–34 (Manitoba Energy and Mines, 1988).
- Leclair, A., Scott, R. & Lucas, S. B. in *Geol. Surv. Can. Pap.* 93–1C, 249–258 (1993).

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Implications of an exceptional fossil flora for Late Cretaceous vegetation

Scott L. Wing*, Leo J. Hickey† & Carl C. Swisher‡

* Department of Paleobiology, National Museum of Natural History, Smithsonian Institution, Washington DC 20560, USA

† Department of Geology and Geophysics, PO Box 6666, 170 Whitney Avenue, Yale University, New Haven, Connecticut 06520, USA

‡ Geochronology Center, Institute of Human Origins, 2453 Ridge Road, Berkeley, California 94709, USA

THE rapid radiation of angiosperms during the Late Cretaceous has been thought to reflect their rise to vegetational dominance^{1–3}. The number of species in a clade and its vegetational importance are not necessarily related, however. Quantitative studies of the recently discovered Big Cedar Ridge flora, found preserved *in situ* in a mid-Maastrichtian volcanic ash in central Wyoming, USA, reveal that dicotyledonous angiosperms accounted for 61% of the species but constituted just 12% of vegetational cover. Dicots, many of which appear to have been herbaceous, were abundant only in areas disturbed just before burial. By contrast, free-sporing plants were 19% of the species but 49% of cover. The only abundant and ubiquitous angiosperm was a single species of palm (about 25% of cover). A comparably low abundance of dicots was found in two other nearly contemporaneous floras buried by volcanic ash, whereas coeval floras from fluvial environments are dominated by dicots⁴. This shows that, even as late as the mid-Maastrichtian, in northern mid-latitudes there were areas away from streams that were not yet dominated by dicots. Despite vigorous taxonomic diversification during the previous 30 Myr³, dicots played a subordinate role in these areas of fern-dominated vegetation.

The Big Cedar Ridge (BCR) fossil flora consists of three-dimensional organic films preserved in the basal 10–20 cm of a 4–5-m-thick bentonitic tuff. When discovered, the tuff had been mapped as part of the Paleocene Fort Union Formation⁵, but

⁴⁰Ar/³⁹Ar analysis of single crystals of euhedral biotite from the basal part of the BCR tuff resulted in a weighted mean age of 71.77 ± 0.16 Myr (Table 1). Subsequently it has been shown that the BCR tuff lies within 8 m of a magnetic polarity reversal correlated biostratigraphically with the top of Chron C32N, which also implies an age of about 71.7 Myr^{6,7} (J. F. Hicks, personal communication). Stratigraphically, the BCR tuff belongs to the Meeteetse Formation, an upper Cretaceous deltaic and coastal plain unit deposited on the western shore of the North American epicontinental seaway during the Campanian and Maastrichtian⁴ (Fig. 1a). Meeteetse palaeoclimate was subtropical and wet^{4,8}.

The BCR tuff overlies a highly variable, generally organic-rich palaeosol formed on muddy to sandy fluvial sediments. These units are exposed in three long outcrops over a total distance of 4 km (Fig. 1b). There is strong evidence for *in situ* preservation of the flora: a number of palmetto and tree fern trunks rooted in the soil project 10–20 cm into the tuff; leaves frequently are found attached to axes and compound fronds are generally articulated; and the composition of the flora changes radically across distances of a few metres.

Five features of the BCR flora show that it was derived from open fern and palmetto-dominated vegetation with minor dicot herbs and scattered conifers, rather than the dicot-dominated, canopied forest that would be expected to occur in a similar climate today (Fig. 1c–e). First, within-site species richness is very high (Table 2), especially considering the assemblage represents an instant in time and the census sites are small. The upper levels of richness recorded at BCR (30–34 spp. per census site) are rare even in the autochthonous litter of diverse tropical forests, because the latter show strong dominance of leaves from a few local canopy species^{9,10}. Second, there is a very high abundance and diversity of pteridophytes (mean of 6.5 species per census; 28 species known from the BCR tuff), which is consistent with a diverse and abundant herb layer. Preliminary examination of the palynoflora shows it too is dominated by ferns. Third, many of the dicot species have leaf features that suggest they were herbs or scramblers (for example, funnel-form bases, deeply cordate bases, or small leaves attached to small upright stems). Fourth, provisional determinations of the dicots

TABLE 1 $^{40}\text{Ar}/^{39}\text{Ar}$ laser total fusion analyses of selected biotite grains from the BCR bentonite of the Meeteetse Formation

Sample	$^{40}\text{Ar}/^{39}\text{Ar}$	$^{37}\text{Ar}/^{39}\text{Ar}$	$^{36}\text{Ar}/^{39}\text{Ar}$	$^{40}\text{Ar}^*/^{39}\text{Ar}$	% $^{40}\text{Ar}^*$	Age (Myr)	$\pm$ s.d.
3601-01	2.2460	0.0300	0.0005	2.1074	93.8	71.765	0.293
3601-02	2.3660	0.0169	0.0009	2.1099	89.2	71.848	0.518
3601-03	2.2691	0.0333	0.0005	2.1073	92.9	71.760	0.767
3601-04	2.4449	0.0042	0.0011	2.1105	86.3	71.868	0.762
3601-05	2.4233	0.0016	0.0011	2.1058	87.0	71.827	0.718
3601-06	2.2121	0.1071	0.0004	2.0978	95.2	71.711	0.464
3601B-01	2.2564	0.0564	0.0005	2.1047	93.3	71.673	1.015
3601B-02	2.2543	0.0247	0.0005	2.1073	93.5	71.761	0.765
3601B-03	2.3018	0.0458	0.0007	2.1032	91.4	71.625	0.441
3601B-05	2.6124	0.0304	0.0017	2.1051	80.6	71.688	0.891
3601B-06	2.4516	0.0073	0.0012	2.1078	86.0	71.779	0.803
3601B-08	2.3253	0.0272	0.0007	2.1110	90.8	71.886	0.429
Weighted mean						71.768	0.155 (s.e.)
Contaminant grains?							
3601B-04	7.4410	0.0058	0.0007	7.2465	97.4	235.658	0.774
3601B-07	2.6111	0.0161	0.0015	2.1699	83.1	73.849	0.552

Analysis procedures followed ref. 15. Ca^{2+} and K^+ corrections were determined from laboratory salts: $(^{36}\text{Ar}/^{37}\text{Ar})_{\text{Ca}} = 2.557 \times 10^{-4} \pm 4.6 \times 10^{-6}$, $(^{39}\text{Ar}/^{37}\text{Ar})_{\text{Ca}} = 6.608 \times 10^{-4} \pm 2.53 \times 10^{-5}$, and $(^{40}\text{Ar}/^{39}\text{Ar})_{\text{K}} = 2.4 \times 10^{-3} \pm 7.0 \times 10^{-4}$. J , the irradiation coefficient ($0.019256 \pm 0.000009 (\pm 0.05\%)$), is based on replicate single-crystal analyses of the monitor mineral Fish Canyon Sanidine, with a reference age of 27.84 Myr as recommended in ref. 16, but slightly modified as a result of in-house intercalibration at the Geochronology Center with MMhb-I with a published age of 520.4 ± 1.7 Myr¹⁷. Mass discrimination during this study, determined by replicate air aliquots delivered from an on-line pipette system, was 1.007 ± 0.002 . Decay constants are from refs 18 and 19. The uncertainties (s.d.) associated with the individual $^{40}\text{Ar}/^{39}\text{Ar}$ ages are 1σ errors; that for the calculated weighted mean age of the replicate analyses is a standard error (s.e.) following ref. 20.

* Radiogenic ^{40}Ar .

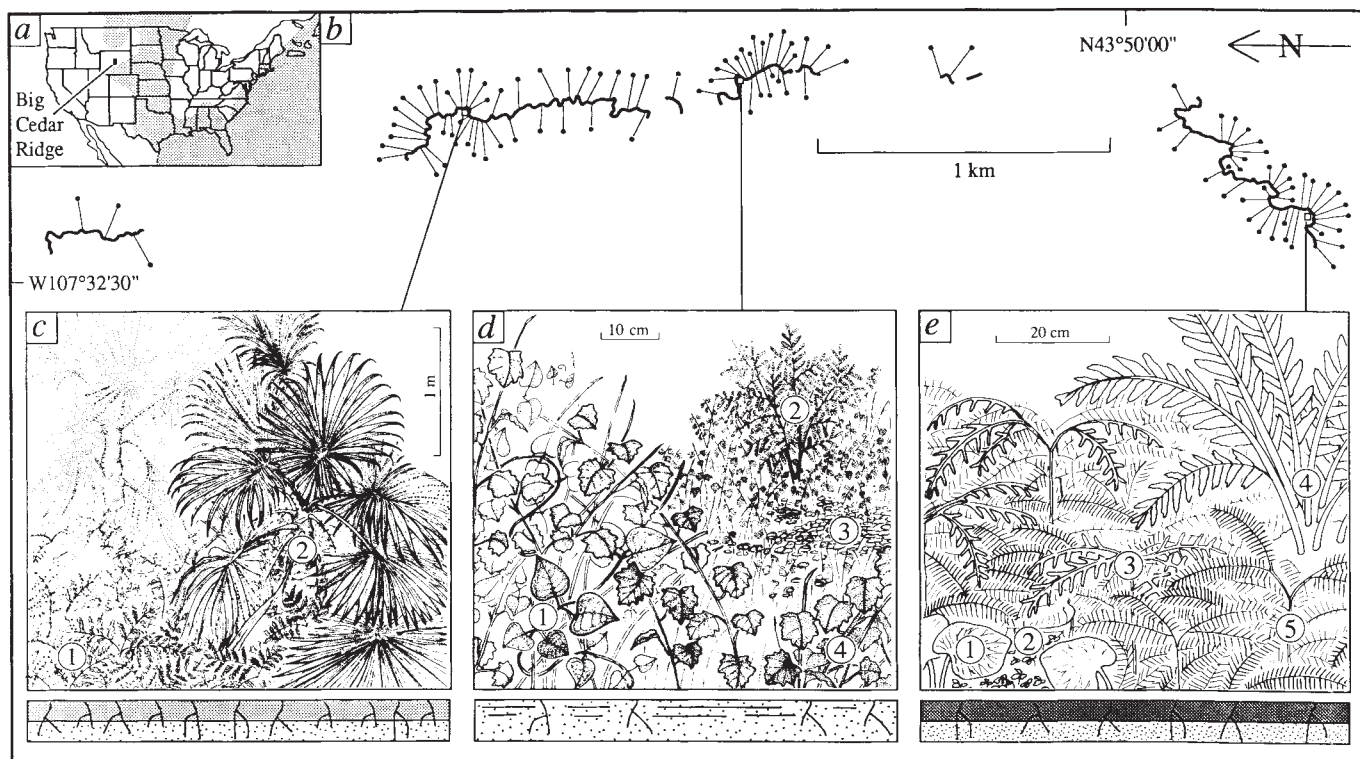


FIG. 1 a, Map of North America shows location of the BCR site with respect to the mid-Maastrichtian (*Baculites grandis* zone) shoreline of the epicontinental seaway; shoreline position from ref. 22. b, Dark line shows outcrop of BCR tuff; small crossing lines show positions of 100 census sites, which were determined largely by accessibility. c-e, Sketches of three types of vegetation found in the BCR tuff, based on outcrop censuses and *in situ* specimens, with soil profiles underneath; scale bars apply to foreground vegetation: c, vegetation on an organic silt substrate dominated by (1) a

fern cf. *Anemia fremontii* and (2) coryphoid palms; d, vegetation of a recently disturbed site on fine sandy substrate dominated by (1) '*Ficus*' *planicostata*, a probable scrambler, (2) an undescribed magnoliid dicot, (3) a peltate-leaved dicot, and (4) cf. '*Acer*' *cretaceum*; e, a peat substrate community with (1) *Hausmannia* (Dipteridaceae), (2) a small dicot similar to *Thalictrum*, (3) an undescribed dipteridaceous or matoniaceous fern, (4) the cycad *Ctenis*, and (5) abundant gleicheniaceae ferns.

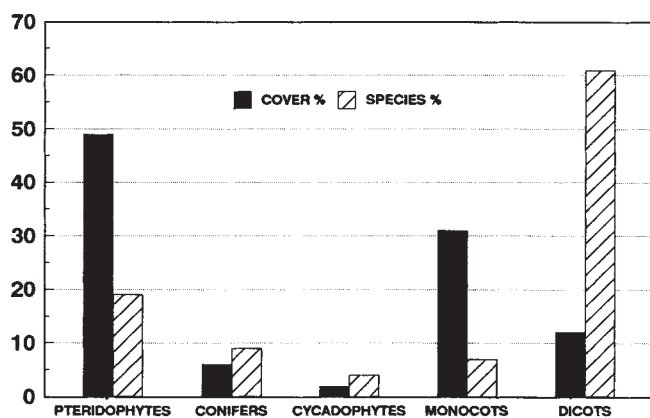


FIG. 2 Comparison of cover and species richness for major groups of plants in the 100 censuses. Pteridophytes (ferns and a few lycopids) and monocots (mostly palms) are abundant but not particularly diverse. Dicots are extremely diverse but not very abundant. See Table 2 legend for data and details of sampling procedure, and Fig. 1b for spacing of localities.

indicate many have affinities with the ranunculid and palaeoherb groups^{11,12}. Fifth, some of the most ubiquitous groups of Late Cretaceous dicot trees (platanoids, cercidiphylloids) are rare in the BCR tuff, suggesting that the ash preserves a type of vegetation not recorded in more typical fluvial depositional environments.

The taxonomic distribution of BCR species is close to the global average for the Maastrichtian¹³: 19% free-sporing lycopids and ferns, 9% conifers, 4% cycadophytes and 68% angiosperms. But comparison of megafossil richness and abundance data at BCR shows no correlation between the two (Fig. 2). Dicots were 61% of the species recovered even though they were relatively uncommon. The mean census richness of dicots (6.9; s.d. = 3.5) is essentially the same as that of ferns, but the ratio of total richness to mean census richness is much higher for dicots (12.9) than for pteridophytes, conifers, cycads and monocots (4.3). These ratios reflect greater heterogeneity in species composition across the landscape for dicots than for other groups; this difference in distribution is consistent with the hypothesis that insect-pollinated dicots were able to exist in highly dispersed populations¹².

At only 3 of the 100 sites did dicots exceed 50% of identified plant cover, and at only 18 did they exceed 30%. At all sites where dicots exceeded 20% of cover there was sedimentological evidence for physical disturbance shortly before the deposition of the BCR tuff: either the underlying soil was relatively coarse, or primary sedimentary structures indicating recent deposition are preserved just below the soil/tuff interface (Fig. 1d).

In spite of evidence for local disturbance in a few areas along the outcrop, the soil underlying the BCR tuff generally is rooted and has high levels of dispersed organic matter or a 4–8 cm coal

TABLE 2 Summary statistics on major plant groups at BCR

	Species per census			Total spp.	Line intercept per census (cm)		
	Mean	Max	Min		Mean	Max	Min
Pteridophytes	6.5	12	0	28	375.6	1,166	0
Conifers	3.0	7	0	13	43.6	444	0
Cycadophytes	0.6	3	0	6	12.0	170	0
Monocots	2.3	4	1	10	240.0	1,127	18
Dicots	6.9	15	0	89	94.8	363	0
Totals	19.3	34	8	146	766.0	1,701	184

All figures are derived from 100 roughly equal-sized censuses along the BCR outcrop (Fig. 1b). A modified line intercept method was used to estimate the area of bedding plane covered by each species; cover is commonly used as a measure of importance in living herbaceous vegetation²¹. Census excavations were roughly square, with a mean size of 3.4 m² (s.d. = 3.0). Fossiliferous tuff was removed as blocks, and all bedding surfaces displaying at least one identifiable plant fragment were included in the census. A frame strung with parallel threads spaced 2 cm apart and marked off in 2-cm segments was placed over each block. All 2-cm segments were recorded as touching a particular species, an unidentifiable plant fragment, or blank rock. If a single segment touched both identifiable and unidentifiable remains, the whole segment was assigned to the identified species. Segments that touched two species were scored 1/2 for each species. If multiple segments touched a single leaf they were all scored for that species. Each census consisted of four replicate samples of 500 segments (10 m of line). The mean amount of intercept per site was 4,190.6 cm (s.d. = 727.4), of which 766 cm (s.d. = 315.8) were identifiable plant fragments. Our tests of this line intercept method on simulated fossil assemblages showed that the relative area of different 'species' of paper leaves could be estimated to within $\pm 2\%$ (manuscript in preparation).

deposit at its top. These features indicate a substantial period of soil formation, and imply that the fern–palm dominated vegetation preserved in the BCR tuff does not represent early primary succession. There is also no fusain concentrated in the upper part of the soil to suggest a fire shortly before the preservational event, nor are there footprints or bioturbation that indicate dinosaur feeding. Furthermore, censuses of plants preserved in two other Meeteetse tuffs of similar age over 100 km away resemble the BCR flora in having low cover of dicots; they are dominated either by ferns or conifers. Together these sites provide direct evidence for the hypothesis¹⁴ that in spite of high species numbers, dicots played a minor role in the vegetation of northern mid-latitude stable land surfaces as late as the mid-Maastrichtian.

The BCR flora reveals the possible pitfalls of inferring ecological dominance from taxonomic lists, as well as the 'mega-biases' associated with a plant fossil record derived mainly from areas disturbed by frequent sedimentation. Quantitative analyses of assemblages such as BCR that represent instantaneous preservation of an entire local vegetation can help calibrate interpretations based on more abundant fluvial assemblages, and greatly amplify our understanding of the ecological setting of plant evolution. □

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- Niklas, K. J., Tiffney, B. H. & Knoll, A. H. in *Phanerozoic Diversity Patterns: Profiles in Macroevolution* (ed. Valentine, J. W.) 97–128 (Princeton Univ. Press, New Jersey, 1985).
- Knoll, A. H. in *Community Ecology* (eds Diamond, J. & Case, T. J.) 126–141 (Harper & Row, New York, 1986).
- Crane, P. R. in *The Origins of Angiosperms and their Biological Consequences* (eds Friis, E. M., Chaloner, W. G. & Crane, P. R.) 107–144 (Cambridge Univ. Press, UK, 1987).
- Hickey, L. J. *Am. J. Bot.* **78**, 115 (1991).
- Love, J. D. & Christiansen, A. C. *Geological Map of Wyoming* (US Geological Survey, 1985).
- Harland, W. B. et al. *A Geologic Time Scale 1989* (Cambridge Univ. Press, UK, 1990).
- Cande, S. C. & Kent, D. V. *J. geophys. Res.* **97**, 13917–13951 (1992).
- Horrell, M. A. *Palaeogeogr. Palaeoclimat., Palaeoecol.* **86**, 87–138 (1991).
- Burnham, R. J., Wing, S. L. & Parker, G. G. *Paleobiology* **18**, 34–53 (1992).
- Greenwood, D. R. *Rev. Paleobot. Palyn.* **71**, 149–190 (1992).
- Taylor, D. W. & Hickey, L. J. *Pl. Syst. Evol.* **180**, 137–156 (1992).
- Doyle, J. A. & Hickey, L. J. in *Origin and Early Evolution of Angiosperms* (ed. Beck, C. B.) 139–206 (Columbia Univ. Press, New York, 1976).
- Lidgard, S. & Crane, P. R. *Paleobiology* **16**, 77–93 (1990).

- Wing, S. L. & Tiffney, B. H. *Rev. Paleobot. Palyn.* **50**, 179–210 (1987).
- Swisher, C. C. et al. *Science* **257**, 954–958 (1992).
- Cebula, G. T. et al. *TERRA Cognita* **6**, 139–140 (1986).
- Samson, S. D. & Alexander, E. C. *Chem. Geol. Isot. Geosci. Sect.* **66**, 27–34 (1987).
- Steiger, R. H. & Jager, E. *Earth planet. Sci. Lett.* **36**, 359–362 (1977).
- Dalrymple, G. B. *Geology* **7**, 558–560 (1979).
- Taylor, J. R. *An Introduction to Error Analysis* (University Science, Mill Valley, California, 1982).
- Greig-Smith, P. *Quantitative Plant Ecology* (Univ. California Press, Berkeley, 1983).
- Lillegraven, J. A. & Ostresh, L. M. Jr in *Dawn of the Age of Mammals in the Northern Part of the Rocky Mountain Interior, North America* (eds Bown, T. M. & Rose, K. D.) 1–30 (Geol. Soc. Am., Special paper **243**, Boulder, 1990).

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A neural basis for visual search in inferior temporal cortex

Leonardo Chelazzi*, Earl K. Miller*, John Duncan† & Robert Desimone*

*Laboratory of Neuropsychology, National Institute of Mental Health, Building 9, Room 1E104, Bethesda, Maryland 20892, USA

†MRC Applied Psychology Unit, 15 Chaucer Road, Cambridge CB2 2EF, UK

WE often search for a face in a crowd or for a particular object in a cluttered environment. In this type of visual search, memory interacts with attention: the mediating neural mechanisms should include a stored representation of the object and a means for selecting that object from among others in the scene¹⁻⁴. Here we test whether neurons in inferior temporal cortex, an area known to be important for high-level visual processing, might provide these components. Monkeys were presented with a complex picture (the cue) to hold in memory during a delay period. The cue initiated activity that persisted through the delay among the neurons that were tuned to its features. The monkeys were then given 2-5 choice pictures and were required to make an eye movement to the one (the target) that matched the cue. About 90-120 milliseconds before the onset of the eye movement to the target, responses to non-targets were suppressed and the neuronal response was dominated by the target. The results suggest that inferior temporal

cortex is involved in selecting the objects to which we attend and foveate.

On each trial, the monkeys first fixated a dot on a computer display. One of two cues was then presented over the dot for 300 ms. The cues were carefully selected for each cell to include a 'good' stimulus that elicited a strong response from the cell and a 'poor' stimulus that elicited little or no response (Fig. 1). After a delay, the good and the poor stimulus were both presented extrafoveally as choice stimuli. The animal made a saccadic eye movement to the target stimulus that matched the cue, ignoring the non-matching stimulus (the distractor). The relative locations of target and distractor varied randomly across trials. All cells were tested in a blocked-trial design, in which one of the two cues was used for a block of 20-30 trials before switching to the other cue, and some were also tested in a more difficult unblocked design, in which the cue varied randomly from trial to trial.

The inferior temporal cells we recorded had activity related to the cue that was maintained throughout the delay, which is consistent with reports of 'delay activity' during delayed matching-to-sample tasks in this area⁵⁻⁷. For 52% of 83 cells recorded in the blocked design, the good cue evoked a significantly higher maintained discharge throughout the delay period than did the poor cue (*t*-test on the last 500 ms of the delay period, $P < 0.05$). The same was true for 38% of 29 cells recorded in the unblocked design, an example of which is shown in Fig. 2.

In addition to information about the cue, inferior temporal cells also communicated information about the target. Figures 2 (single cell) and 3 (population of cells) show the responses to choice arrays (in the contralateral hemifield) that were physically identical but in which either the good or poor stimulus

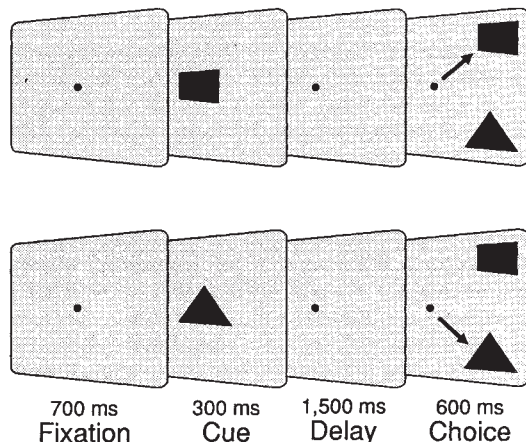


FIG. 1 Sequence of stimulus events. Before the experimental trials, we tested each cell with a variety of 1-1.5 deg² complex patterns (digitized magazine pictures) presented at the centre of gaze, and selected a 'good' stimulus and a 'poor' stimulus, represented here for simplicity by the square and triangle. Each trial began with a cue followed, after a 1,500-3,000-ms delay, by two choice stimuli presented peripherally. The animal made an eye movement (arrow) to the target that matched the cue in order to receive juice reward. Stimuli were extinguished 150 ms after the animal fixated the target. In other trials (data not shown), neither choice stimulus matched the cue and the animal was rewarded for maintaining initial fixation. The critical comparison was the neuronal response to choice arrays that contained the same stimuli (triangle and square, for example), but in which either the good or poor stimulus was the target. If, for example, a cell responded only to squares and not to triangles, we compared the response to the choice array preceded by the square (upper panel) to the response to the same array preceded by the triangle (lower panel). Target and distractor were typically located at an eccentricity of 4-5° and were separated by the vertical or horizontal meridian. In the former configuration, they were centred in the lower quadrants of each hemifield, and in the latter configuration one was centred in the upper field and one in the lower. Relative locations of target and distractor in each configuration were varied randomly. Cells generally responded best to stimuli presented at the centre of gaze, less well to stimuli in the contralateral field and weakly (in some cases not at all) to stimuli in the ipsilateral field.

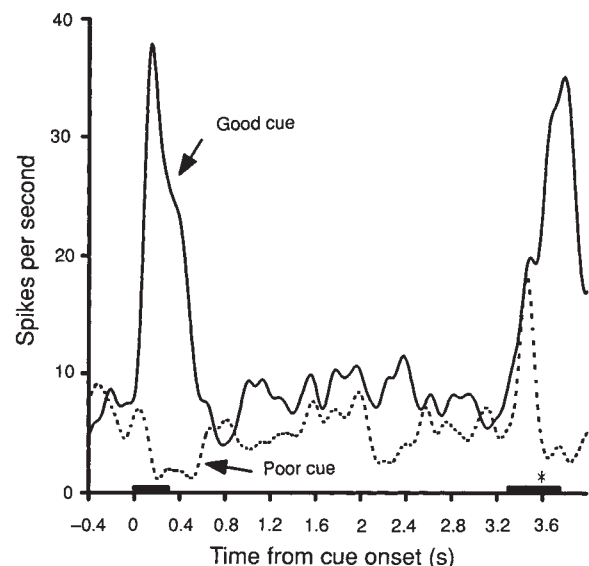
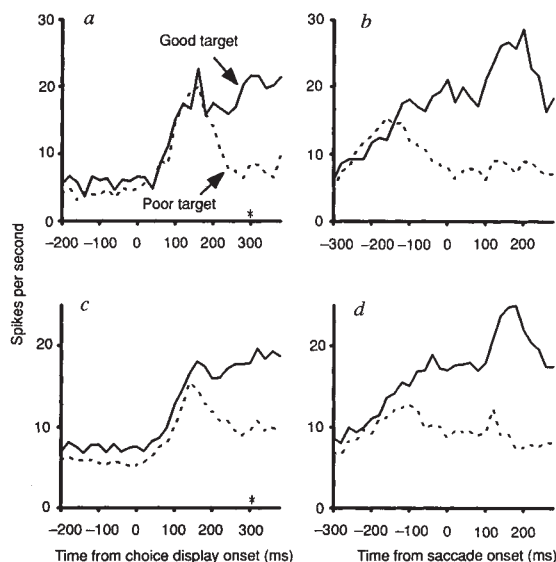


FIG. 2 Standard electrophysiological and eye monitoring techniques were used to record from cells in two monkeys (*Macaca mulatta*)^{7,8}. Magnetic resonance imaging was used to place the recording electrodes into the anterior inferior temporal cortex, between the anterior middle temporal and the rhinal sulci. Graphs show average firing rates of one cell on trials with either good or poor stimulus presented as cue. The good stimulus elicited a strong excitatory response and the poor stimulus was slightly inhibitory. Firing rates in each 10-ms bin were convolved with a gaussian with a standard deviation of 40 ms¹³. Each frequency plot represents the average of 20 trials. Black horizontal bars indicate presentation of cue and choice stimuli. Average time of saccade onset to the target, 297 ms after array onset, is indicated by an asterisk. The average firing rates in the last 500 ms of the delay for all cells showing significantly different activity following the good versus poor cue, were 7.9 versus 5.6 spikes per second, respectively, in the blocked design, and 6.8 versus 4.0 spikes per second, respectively, in the unblocked.



was the target. Especially in the blocked design, the elevated activity in the delay following the good cue persisted into the initial neuronal response to the choice array. Relative to the preceding delay activity, however, this initial response to the array was about the same regardless of which choice stimulus was the target. By contrast, the late phase of the response changed dramatically, depending on whether the animal was about to make a saccade to the good or poor stimulus. If the target was the good stimulus, the response remained high, but if the target was the poor stimulus, the response to the good distractor stimulus was suppressed even though it was still within the receptive field. It is as though 90–120 ms before the start of the saccade (~200 ms after choice array onset), the target stimulus ‘captured’ the response of the cell, so that neuronal activity would reflect only the target’s properties.

In the 90-ms period before saccade onset, 78% (blocked) and 91% (unblocked) of the cells gave a larger response when the good stimulus was the target, out of the 58 and 22 cells, respectively, that responded to the extrafoveal choice array. The larger response when the good stimulus was the target was significant for 48% (blocked) and 45% (unblocked) of all cells according to a *t*-test ($P < 0.05$) performed separately on each cell. Only one cell showed a significant effect in the opposite direction. Previous studies have shown that when animals are cued to attend to stimuli at one location and ignore stimuli at another, inferior temporal responses to the ignored stimuli are suppressed^{8,9}; however, this is the first evidence for such suppression when the location of the attended stimulus is not known

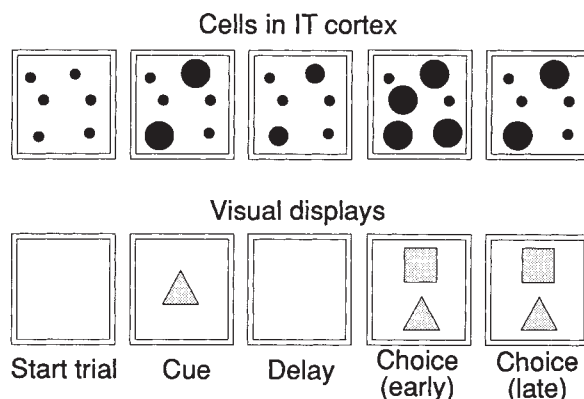
FIG. 3 Average firing rates to identical choice arrays, in which either the good (solid lines) or poor (dashed lines) stimulus was the target (that is, preceded by good or poor stimulus as cue). Graphs show the average response of all cells that responded to choice arrays (58 in the blocked design and 22 in the unblocked). The bin width is 20 ms. *a*, Responses of cells in the unblocked design, time-locked to array onset. Average time of saccade onset, 300 ms after array onset, is indicated by an asterisk. *b*, Same data as in *a*, but responses are time-locked to saccade onset. *c*, Responses of cells in the blocked design, time-locked to array onset. The average saccade latency in this design was 306 ms. *d*, The same data as in *c*, but time-locked to saccade onset. Data in all graphs are from trials in which the target and distractor appeared in the hemifield contralateral to the recorded cell. Data are averaged from trials in which the target was in the upper quadrant and the distractor in the lower, and vice versa. The target effect was much smaller when target and distractor were located in opposite hemifields, a finding which is consistent with those of Sato⁹ and of J. Moran and R.D. (unpublished data), who found reduced spatial attentional effects in inferior temporal cortex when attended and ignored stimuli were in opposite hemifields. With the choice array confined to the contralateral field, 33% (blocked) and 50% (unblocked) of the cells gave significantly different responses to the array, depending on the relative locations of the good and poor stimuli. Thus some spatial (retinotopic) information is retained in inferior temporal cortex in spite of the large receptive fields.

in advance and object features themselves guide selection.

To test whether the target-selection results would generalize to larger search displays, ten additional cells were studied in one monkey in the same task, but with extrafoveal choice arrays consisting of five stimuli arranged in a semicircle within the contralateral field. All ten showed the same pattern of results as the cells tested with two-stimulus arrays: if the target on a given trial was a good stimulus for the cell, the response remained high, but if the target was a poor stimulus, the response was suppressed beginning 90–120 ms before the saccade, even though the good stimulus was still within the receptive field. Saccadic latencies increased by 26 ms per stimulus, with choice arrays of 1, 2, 3 and 5 stimuli.

Figure 4 illustrates that both of the components necessary for this type of visual search—internal representation of cue and selection of target—are reflected in the responses of inferior temporal neurons. It remains to be determined whether these two components are causally linked, that is, whether it is the maintained activity initiated by the cue or some other mechanism (such as interaction with separate attentional systems) that ultimately results in suppression of inferior temporal responses to non-targets. The possibility of a link is supported by the fact that more than three-quarters of the cells with significant target effects in the 90 ms preceding a saccade also showed significant cue-initiated delay effects. In either case, target information in inferior temporal cortex is available to drive oculomotor systems, resulting in eye movements to the chosen object^{10–12}. More generally, networks in inferior temporal cortex may select the

FIG. 4 Upper diagrams are schematic representations of activity in a population of inferior temporal (IT) neurons during performance of the task. Each dot represents an individual cell, and the size of the dot indicates relative firing rate. Lower diagrams illustrate the visual displays during the relevant portions of the task. A specific cue activates the subpopulation of IT cells tuned to any of the various features of the cue. During the delay period, this subpopulation remains more active than other cells. When the choice array first appears, cells are initially activated by whichever stimulus they prefer in the array, regardless of which is the target. In some cases the initial responses are identical, but in others the response to the array with the good target starts off at a higher rate because of persisting activity from the delay. Later, within 90–120 ms of saccade onset, the cells tuned to the properties of the target stimulus remain active, whereas cells tuned to the properties of the distractor are suppressed. Whether this final divergence in activation results from competitive interactions within IT cortex (for example, through mutual inhibition between cells selective for target and distractor), or from interactions between IT cortex and an attentional control system, is not yet known.



visual objects that are acted upon by motor systems when selection is guided by object features. □

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1. Neisser, U. *Am. J. Psych.* **76**, 316–385 (1963).
2. Treisman, A. Q. *J. exp. Psych.* **40A**, 201–237 (1988).
3. Duncan, J. & Humphreys, G. W. *Psych. Rev.* **96**, 433–458 (1989).
4. Bundesen, C. *Psych. Rev.* **97**, 523–547 (1990).
5. Fuster, J. M. & Jervey, J. P. *Science* **212**, 952–955 (1981).
6. Miyashita, Y. & Chang, H. S. *Nature* **331**, 68–70 (1988).

7. Miller, E. K., Li, L. & Desimone, R. *J. Neurosci.* **13**, 1460–1478 (1993).
8. Moran, J. & Desimone, R. *Science* **229**, 782–784 (1985).
9. Sato, T. *J. Neurophysiol.* **60**, 344–364 (1988).
10. Schiller, P. H. & Koerner, F. *J. Neurophysiol.* **34**, 920–936 (1971).
11. Wurtz, R. H. & Goldberg, M. E. *J. Neurophysiol.* **35**, 575–586 (1972).
12. Glimcher, P. W. & Sparks, D. L. *Nature* **355**, 542–545 (1992).
13. Richmond, B. J., Optican, L. M. & Spitzer, H. *J. Neurophysiol.* **57**, 132–146 (1987).

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Induction of LTP in the hippocampus needs synaptic activation of glutamate metabotropic receptors

Zafar I. Bashir, Zuner A. Bortolotto, Ceri H. Davies, Nicola Berretta, Andrew J. Irving, Andrew J. Seal, Jeremy M. Henley, David E. Jane*, Jeffrey C. Watkins* & Graham L. Collingridge

Department of Pharmacology, The Medical School, University of Birmingham, Edgbaston, Birmingham B15 2TT, UK
* Department of Pharmacology, The School of Medical Sciences, University of Bristol, Bristol BS8 1TD, UK

UNDERSTANDING the mechanisms of long-term potentiation (LTP) should provide insights into the molecular basis of learning and memory in vertebrates. Ionotropic glutamate receptors play a central role in LTP; AMPA (α -amino-3-hydroxy-5-methyl-4-isoxazolepropionate) receptors and NMDA (*N*-methyl-D-aspartate) receptors mediate synaptic responses that are enhanced in LTP and, in addition, NMDA receptors are necessary for the induction of LTP in most pathways¹. There is also circumstantial evidence that metabotropic glutamate receptors (mGluRs) may be involved in LTP because the specific mGluR agonist aminocyclopentane dicarboxylate can augment tetanus-induced LTP² and, under certain circumstances, can itself induce a slow-onset potentiation^{3,4}. But the absence of any effective mGluR antagonist has prevented the determination of whether mGluRs are involved in the induction of tetanus-induced LTP. We report here that (*RS*)- α -methyl-4-carboxyphenylglycine is a specific mGluR antagonist in the hippocampus and have used this compound to examine the nature of the involvement of mGluRs in LTP. We show that synaptic activation of mGluRs is necessary for the induction of both NMDA receptor-dependent and NMDA receptor-independent forms of LTP in the hippocampus.

Establishing a physiological role for mGluRs in LTP requires the use of specific mGluR antagonists. The involvement of mGluRs in LTP has been suggested on the basis of the actions of the drugs aminophosphonobutanoate (AP4)⁵ and aminophosphonopropionate (AP3)^{6–8}. But these compounds do not affect mGluR-mediated responses in the hippocampus, as determined electrophysiologically^{9–12}. Moreover, the uncompetitive nature of the actions of AP3 and AP4 on metabolic manifestations of mGluR activity makes it difficult to interpret their effects on LTP. Recently, however, it has been reported that (*RS*)- α -methyl-4-carboxyphenylglycine (MCPG) is a selective and competitive mGluR antagonist with respect to aminocyclopentane dicarboxylate (ACPD)-stimulated phosphoinositide hydrolysis and ACPD-induced electrophysiological responses in rat brain and spinal cord¹³. To confirm the effectiveness of MCPG as an mGluR antagonist, we first tested it on a system devoid of ionotropic glutamate receptors. MCPG (500 μ M) reversibly abolished Ca^{2+} -mobilizing responses induced by the

stimulation of mGluR1 α -transfected cells¹⁴ by 1S,3R-ACPD, an active enantiomer of ACPD¹⁵ (Fig. 1a). We next determined its effectiveness as an mGluR antagonist in the hippocampus. In CA1 neurons, MCPG (500 μ M) reversed the inhibition by 1S,3R-ACPD¹⁶ of both action potential accommodation and the ensuing after-hyperpolarization (AHP) (Fig. 1b). In contrast, MCPG (tested at 1,000 μ M) had no effect on the inhibition by carbachol of action potential accommodation and the AHP (Fig. 1c). We also tested the effects of MCPG on the four components of the synaptic response that can be elicited by low-frequency stimulation of the Schaffer collateral-commissural pathway. MCPG (500 μ M) had no effect on either of the γ -aminobutyric acid (GABA_A or GABA_B) receptor-mediated components of inhibitory postsynaptic potentials (i.p.s.ps) (Fig. 2a), NMDA receptor-mediated excitatory postsynaptic currents (e.p.s.cs) (Fig. 2b) or AMPA receptor-mediated excitatory postsynaptic potentials (e.p.s.ps) (Fig. 2c). MCPG did, however, block reversibly the generation of slow-onset synaptic potentiation⁴ induced by 1S,3R-ACPD (Fig. 2c). These data show that MCPG is sufficiently potent and selective to be used as an mGluR antagonist to study the physiological roles of mGluRs in the hippocampus.

AP3 has been reported to have variable effects on the induction of LTP in the CA1 region, ranging from no effect¹⁷ to complete block⁶ with D,L-AP3 and blockade of a later component of LTP with L-AP3⁷. We did similar experiments using L-AP3 (1,000 μ M) but have been unable to block LTP in area CA1 (followed for up to 3 h after the tetanus) (Fig. 3a). In contrast, MCPG (500 μ M) reversibly blocked the induction of LTP without affecting basal synaptic transmission or established LTP (Fig. 3b, c). In each of the 5 slices tested, there was short-term potentiation (STP) lasting about 30 min, but never LTP. These data show, therefore, that the synaptic activation of mGluRs is necessary for the induction of LTP in the CA1 region of the hippocampus. Because NMDA receptors are also necessary for the induction of LTP in this region of the hippocampus¹⁸, ionotropic and metabotropic glutamate receptors serve as complementary but essential triggers for LTP induction.

In the CA3 region of the hippocampus, LTP in the mossy fibre pathway does not involve NMDA receptors¹⁹ and no trigger for induction has been positively identified. We therefore tested MCPG on mossy fibre LTP. MCPG (500 μ M) had no effect on basal synaptic transmission but reversibly blocked the induction of NMDA-receptor-independent LTP (Fig. 4). In contrast to area CA1, there was no STP in the presence of MCPG; tetanic stimulation resulted only in post-tetanic potentiation (PTP) which lasted for less than 5 min.

These data provide compelling evidence that the synaptic activation of mGluRs is necessary for the induction of LTP. The failure, in the present study, of L-AP3 to block LTP in area CA1 is not inconsistent with this finding because L-AP3 is, at best, a very weak mGluR antagonist of physiological responses in this region^{9–12}. In an earlier report⁷, L-AP3 reduced the duration of LTP to between 2 and 3 h. It is possible that this difference reflects an effect of L-AP3 on processes involved in

a later component of LTP that was not generated in our experiments. In contrast to L-AP3, D,L-AP3 has been reported to block LTP from its onset⁶; this effect can be explained by the NMDA receptor antagonist properties resident in the D-isomer⁶. AP4 has also been reported to reduce the duration of LTP⁵, but the effects are not stereoselective and are still observed if the drug is applied after the tetanus, observations that are difficult to reconcile with antagonism of the synaptic activation of mGluRs. In contrast to the results with AP3 and AP4, the actions of MCPG are entirely consistent with specific blockade of mGluRs. Although MCPG blocked the induction of LTP, it did not affect synaptic responses induced by low-frequency stimulation of either the Schaffer collateral-commissural or mossy fibre pathways. This implies that any activation of mGluRs by L-glutamate released during low-frequency stimulation has little, if any, direct influence on synaptic transmission.

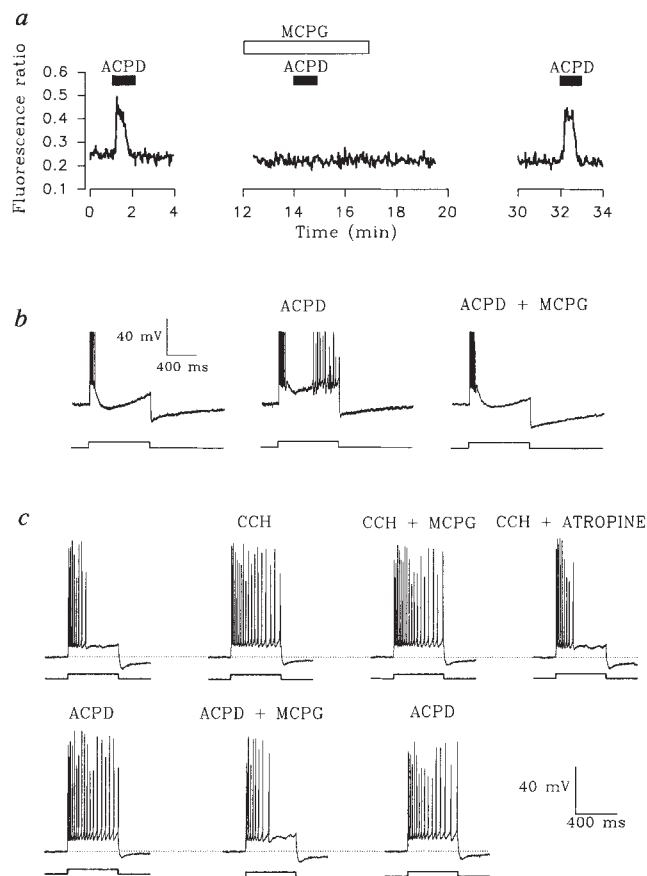
The synaptic activation of NMDA receptors in the CA1 region is necessary for both STP and LTP, because NMDA antagonists block both processes¹⁸. Because the application of NMDA usually only induces STP^{18,20}, it has been suggested that additional triggers may be required for the conversion of STP into LTP²⁰. The present observation that MCPG blocks the induction of LTP, but spares STP, suggests that mGluRs could serve this function. Consistent with this notion, stimulation of mGluRs

with 1S,3R-ACPD can induce a slow-onset potentiation which resembles LTP, without the STP component, and occludes with tetanus-induced LTP⁴. Metabotropic GluRs presumably activate the kinases that are required for the conversion of STP to LTP²¹⁻²³ through their coupling to, for example, phospholipase C (refs 14, 24, 25). In addition to the direct activation of these enzyme systems, mGluRs could aid the generation of LTP by augmenting the activation of NMDA receptors²⁶⁻²⁸ and thereby enhancing postsynaptic Ca^{2+} entry²⁹. Irrespective of the precise mechanism, NMDA receptors and mGluRs provide complementary triggers for the induction of LTP in the CA1 region.

In the mossy fibre pathway, LTP can be induced in the presence of both NMDA and AMPA receptor antagonists⁸. Thus, although the synaptic activation of ionotropic glutamate receptors may augment the effectiveness of the mGluR system^{30,31}, it seems that the participation of ionotropic glutamate receptors is not essential for the induction of LTP. In this pathway, therefore, it is likely that mGluRs provide the principal trigger for the induction of LTP. The involvement of mGluRs, however, does not exclude other receptors, such as opioid receptors³²⁻³⁴, which respond to other neurotransmitters/modulators, which may be released in addition to L-glutamate following stimulation of this pathway. Another difference between CA1 and mossy fibre LTP is the absence of MCPG-resistant

FIG. 1 MCPG selectively blocks 1S,3R-ACPD-induced actions. **a**, Ca^{2+} -mobilizing responses to 50 μM 1S,3R-ACPD in an mGluR1 α -transfected cell were blocked reversibly by 500 μM MCPG. Similar results were obtained in all 16 cells tested in 4 separate experiments. **b**, Reduction of action potential accommodation and the ensuing AHP in a CA1 neuron induced by 30 μM 1S,3R-ACPD is reversed by 500 μM MCPG. Traces are averages of two consecutive records. The neuron was held at -67 mV and depolarizing steps (0.35 nA for 800 ms) were applied through the intracellular electrode. In 5 neurons 30–50 μM 1S,3R-ACPD reduced the peak of the AHP to $47 \pm 2\%$ of control. In these cells MCPG (500 or 1,000 μM) reversed the effect such that the AHP was restored to $97 \pm 8\%$ of control values. **c**, MCPG did not affect carbachol (CCH)-induced reduction in accommodation and AHP. Traces show (from left to right, top row) control response to a depolarizing step (0.5 nA for 500 ms), reduction of accommodation and of the AHP induced by carbachol (1 μM), lack of reversal by MCPG (1,000 μM) but reversal by atropine (1 μM) and (bottom row) reduction of accommodation and the AHP induced by 1S,3R-ACPD (50 μM), reversal by MCPG (1,000 μM) and partial recovery after washout of MCPG. The cell was maintained at -66 mV. MCPG (1,000 μM) did not reverse the effects of carbachol in each of three cells tested. In these experiments 1S,3R-ACPD either caused a small depolarization (of less than 4 mV) or had no effect. Changes in membrane potential were compensated by adjusting the level of DC current injected (this was always less than 0.1 nA).

METHODS. For the Ca^{2+} -imaging experiments, Chinese hamster ovary (CHO) cells permanently expressing mGluR1 α ¹⁴ were cultured on glass coverslips in glutamate-free DMEM supplemented with 10% fetal calf serum and antibiotics. After growth for 1–2 days, cells were incubated for 30 min in perfusion buffer, which comprised (in mM) NaCl, 130; HEPES, 10; KCl, 5.4; CaCl_2 , 1.8; MgCl_2 , 1.0; D-glucose 25, (pH 7.4) and contained 2 μM Indo-1AM. After the loading of dye, cells were incubated for a further 30 min in buffer lacking Indo-1AM to allow de-esterification to occur. Cells were then visualized using an ultraviolet laser-scanning confocal imaging system (BioRad MRC-600). Intracellular Ca^{2+} was monitored ratiometrically in response to the application of 50–100 μM 1S,3R-ACPD in the absence and presence of 500 μM MCPG. No responses to 100 μM 1S,3R-ACPD or 100 μM L-glutamate were detected (36 cells tested in 6 separate experiments) in cells that had been transfected with vector not containing the receptor complementary DNA. For the electrophysiology experiments, rat hippocampal slices (400 μm) were prepared (from 4–6-week-old rats) using standard techniques and perfused with a medium (bubbled with 95% O_2 /5% CO_2) containing (in mM): NaCl, 124; KCl, 3; NaHCO_3 , 26; NaH_2PO_4 , 1.25; CaCl_2 , 2; MgSO_4 , 1; D-glucose, 10. Extracellular field potentials from stratum radiatum of area CA1 or stratum lucidum of area CA3 and intracellular and whole-cell patch-clamp recordings from stratum pyramidale of area CA1 were obtained using electrodes containing 4M NaCl, 3M potassium methylsulphate and (in mM) Cs^+ methanesulphonate, 130; NaCl, 5; CaCl_2 , 1; HEPES, 5; EGTA, 1, pH 7.2, respectively. Patch-clamp experiments were performed at $\sim 20^\circ\text{C}$; all other experiments were done at $\sim 30^\circ\text{C}$. The Schaffer collateral-commissural pathway and mossy fibre pathways were stimulated using shocks delivered



at 30 s (single input experiments) or 60 s (two input experiments in area CA1) intervals. High-frequency (tetanic) stimulation comprised a single period of stimulation at 100 Hz delivered for 1 s, at the test intensity (indicated by an arrow). Synaptic records are the average of four consecutive responses and the stimulus artefacts were blanked for clarity. All drugs were added to the perfusing medium and the timing of drug applications is indicated by bars. Each *n* value applies to the number of times a result was obtained, which is the same as the number of slices tested (each slice was obtained from a separate rat).

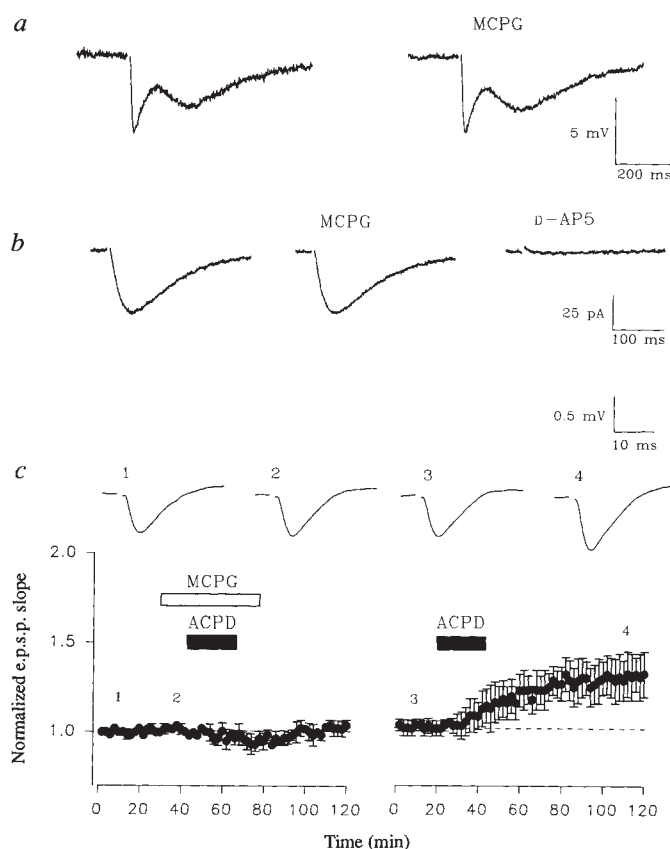
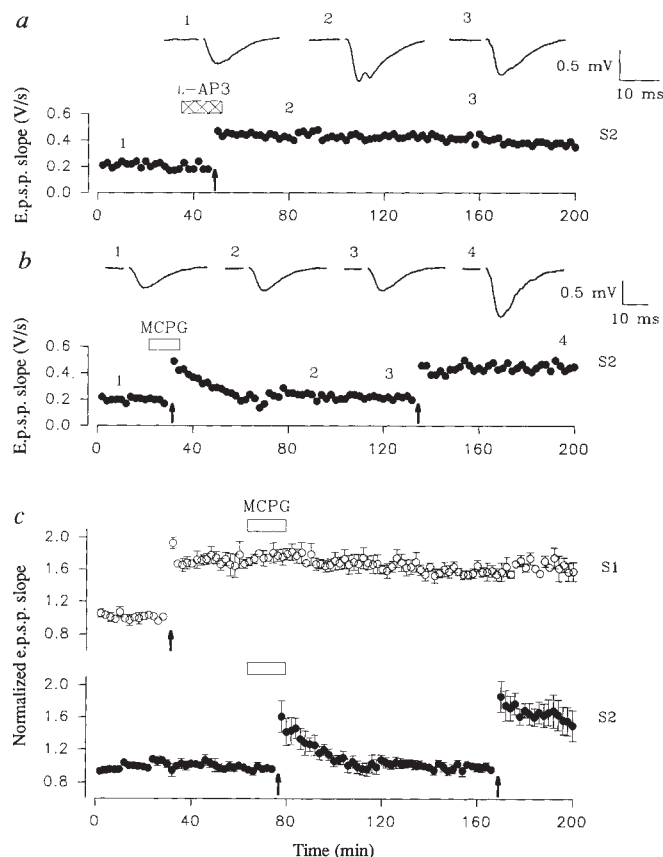


FIG. 2 The effects of MCPG on synaptic responses in the hippocampus. *a*, MCPG (500 μ M) had no effect on monosynaptic GABA-mediated i.p.s.p.s, isolated pharmacologically using the selective AMPA and NMDA receptor antagonists 6-cyano-7-nitroquinoxaline-2,3-dione and D-2-amino-5-phosphonopentanoate (CNQX; 20 μ M and D-AP5; 50 μ M), respectively³⁶. Input impedance (tested using -0.5 nA, 500 ms pulses) and resting membrane potential (-67 mV) did not change. Similar results were observed in each of four cells. *b*, NMDA receptor-mediated synaptic transmission in hippocampal CA1 neurons is not affected by 500 μ M MCPG. The traces are whole-cell recordings of NMDA receptor-mediated synaptic currents (at -60 mV) isolated by using 2,3-dihydroxy-6-nitro-7-sulphamoyl-benzo(F)quinoxaline (NBQX; 5 μ M), picrotoxin (50 μ M) and intracellular Cs^+ to block AMPA, GABA_A and GABA_B receptor-mediated synaptic transmission, respectively. Similar results were obtained in each of four cells. Subsequent application of 50 μ M D-AP5 to 2 of these cells eliminated the synaptic current. MCPG had no effect on input resistance or holding current (not illustrated). *c*, Slow-onset potentiation induced by 10 μ M 1S,3R-ACPD in the CA1 region of the hippocampus is prevented reversibly by 500 μ M MCPG. The graph illustrates pooled data from three separate experiments. Each point on the graph is the average of four consecutive records. Synaptic records, taken from one of these experiments, show the lack of effect of MCPG on the field e.p.s.p. (primarily an AMPA receptor-mediated response) but its ability to block 1S,3R-ACPD-induced slow-onset potentiation.

FIG. 3 Antagonism of mGluRs blocks the induction of tetanus-induced LTP in the CA1 region of the hippocampus. Two separate inputs, designated S1 and S2, were stimulated alternately (with 30 s separating the stimulation of each input). Tetanic stimulation was applied to one of the pathways (S1) and this resulted in LTP (not shown in *a* or *b*) and after 30 min either L-AP3 (1,000 μ M) or MCPG (500 μ M) were applied for 15 min. *a*, L-AP3 had no effect on the potentiated (not shown) or the non-potentiated pathways. Tetanic stimulation applied to pathway S2 in the presence of L-AP3 resulted in LTP (followed for up to 180 min). Similar results were obtained in each of three slices tested. *b*, Tetanic stimulation delivered to S2 in the presence of MCPG resulted in STP, which recovered to baseline within about 30 min. After washout of MCPG, a second tetanus applied to S2 resulted in LTP (followed for up to 90 min). *c*, Pooled data ($\pm$ s.e.m.) showing that 500 μ M MCPG has no effect on potentiated or control synaptic responses, while reversibly blocking the induction of LTP. The graph is composed of data from each of the four control (S1) and five test (S2) pathways tested with MCPG.



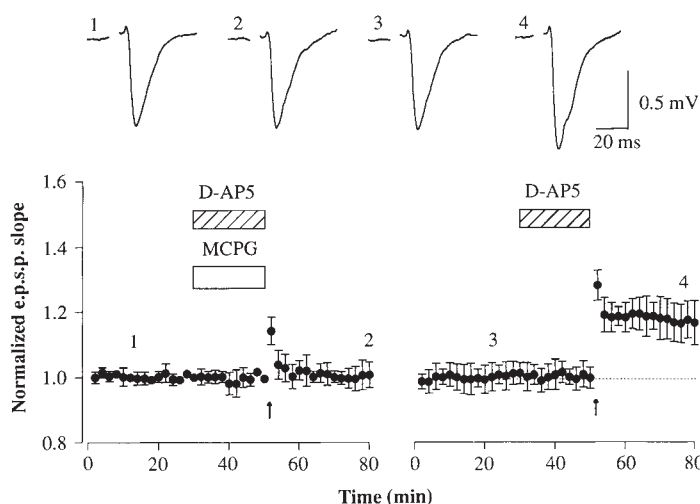


FIG. 4 Antagonism of mGluRs blocks the induction of LTP in the mossy fibre pathway of area CA3. Pooled data ($n=5 \pm \text{s.e.m.}$) showing that tetanic stimulation delivered in the presence of 50 μM D-AP5 and 500 μM MCPG failed to induce LTP. After the washout of MCPG, a second tetanus in the presence of D-AP5 resulted in the induction of NMDA receptor-independent LTP in each example (followed for up to 50 min).

STP in the latter pathway. These differences in the nature of the involvement of mGluRs in the two pathways may reflect the subtypes of mGluRs involved¹⁴, a possibility that cannot be verified until subtype-specific mGluR antagonists are developed.

The synaptic activation of mGluRs provides a necessary trigger for the induction of LTP in both the NMDA receptor-dependent and NMDA receptor-independent forms of LTP in the hippocampus. □

Potential of developing neuromuscular synapses by the neurotrophins NT-3 and BDNF

Ann M. Lohof, Nancy Y. Ip* & Mu-ming Poo†

Department of Biological Sciences, Columbia University, New York, New York 10027, USA

* Regeneron Pharmaceuticals Inc., Tarrytown, New York 10591, USA

THE neurotrophins are a family of neurotrophic factors that promote survival and differentiation of various neuronal populations^{1,2}. Although the long-term effects of neurotrophins on neuronal survival and differentiation have been intensively studied, nothing is known about their effects on synaptic function. Here we report that acute exposure to neurotrophin-3 (NT-3)^{3,4} or brain-derived neurotrophic factor (BDNF)⁵, but not nerve growth factor (NGF)⁶, rapidly potentiates the spontaneous and impulse-evoked synaptic activity of developing neuromuscular synapses in culture. The effect appears to be presynaptic in origin and to be mediated by the Trk family of receptor tyrosine kinases⁷. These results provide evidence for the regulation of the function of developing synapses by neurotrophins.

Developing neuromuscular synapses between *Xenopus* spinal neurons and myotomal myocytes were studied in 1-day-old cell cultures. In the first set of experiments, spontaneous synaptic currents (SSCs) were monitored from innervated myocytes by whole-cell voltage-clamp recording. Bath application of NT-3 (50 ng ml⁻¹) resulted in a marked increase in the frequency of SSCs (Fig. 1a). On average, the frequency increased to 7.8 ± 2.0 (s.e., $n=16$) times the control SSC frequency before the treatment (Fig. 2a). The effect of NT-3 on spontaneous synaptic activity was concentration-dependent (Fig. 1b). The effective concentration of NT-3 was within the range most likely to activate TrkC receptors, and this neurotrophin concentration promotes neuronal survival and neurite outgrowth in other culture preparations⁸⁻¹⁰. The effect of NT-3 on SSC frequency appears to be due to a change in spontaneous acetylcholine (ACh) release from the presynaptic neurons, which is independent of presynaptic action potentials^{11,12}. The distribution of SSC amplitudes remained unchanged after treatment with NT-3 (Fig. 1c), suggesting that the increase in SSC frequency reflects an increased probability of spontaneous ACh secretion rather than an increase in the postsynaptic ACh receptor sensitivity, which might have elevated the amplitude of pre-existing small events above the noise.

The increase in SSC frequency produced by NT-3 was rapid, beginning ~5–10 min after the onset of NT-3 application. The effect was dependent on the continued presence of the factor, because the SSC frequency was reduced almost to the control level when the NT-3-containing medium was replaced with fresh medium after 15 min exposure to NT-3 (Fig. 1d). The effect of a longer treatment (60 min) with NT-3 was tested by recording repeatedly from the same synapse during a control period, during NT-3 incubation, and after washing with fresh culture medium. In these experiments the SSC frequency also returned to the control level after removal of NT-3 (Fig. 1e). The rapid onset of potentiation following NT-3 addition and the immediate decay of potentiation after NT-3 removal suggest that the intracellular effector pathway does not involve a permanent alteration in the secretory machinery, which might have been associated with a traditional differentiation effect of neurotrophins. Nevertheless, the initial step in the synaptic potentiation by NT-3 is likely to be mediated by the same receptors that are responsible for differentiation and survival effects (see below).

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- Bliss, T. V. P. & Collingridge, G. L. *Nature* **361**, 31–39 (1993).
- McGuinness, N., Anwyl, R. & Rowan, M. *Eur. J. Pharmacol.* **197**, 231–232 (1991).
- Zheng, F. & Gallagher, J. P. *Neuron* **9**, 163–172 (1992).
- Bortolotto, Z. A. & Collingridge, G. L. *Neuropharmacology* **32**, 1–9 (1993).
- Reymann, K. G. & Matthies, H. *Neurosci. Lett.* **98**, 166–171 (1989).
- Izumi, Y., Clifford, D. B. & Zorumski, C. F. *Neurosci. Lett.* **122**, 187–190 (1991).
- Behnisch, T., Fjodorow, K. & Reymann, K. G. *NeuroReport* **2**, 386–388 (1991).
- Ito, I. & Sugiyama, H. *NeuroReport* **2**, 333–336 (1991).
- Stratton, K. R., Worley, P. F. & Baraban, J. M. *Eur. J. Pharmacol.* **186**, 357–361 (1990).
- Chapman, S. & Gahwiler, B. H. *Proc. R. Soc. B* **243**, 221–226 (1991).
- Hu, G.-Y. & Storm, J. F. *Acta physiol. scand.* **145**, 187–191 (1992).
- Desai, M. A., Smith, T. S. & Conn, J. P. *Synapse* **12**, 206–213 (1992).
- Eaton, S. A. *et al. Eur. J. Pharmacol.* **244**, 195–197 (1993).
- Aramori, I. & Nakanishi, S. *Neuron* **8**, 757–767 (1992).
- Irving, A. J., Schofield, G., Watkins, J. C., Sunter, D. C. & Collingridge, G. L. *Eur. J. Pharmacol.* **186**, 363–365 (1990).
- Stratton, K. R., Worley, P. F. & Baraban, J. M. *Eur. J. Pharmacol.* **173**, 235–237 (1989).
- Stanton, P. K., Chattarji, S. & Sejnowski, T. J. *Neurosci. Lett.* **127**, 61–66 (1991).
- Collingridge, G. L., Kehrl, S. J. & McLennan, H. *J. Physiol., London* **334**, 33–46 (1983).
- Harris, E. W. & Cotman, C. W. *Neurosci. Lett.* **70**, 132–137 (1986).
- Kauer, J. A., Malenka, R. C. & Nicoll, R. A. *Nature* **334**, 250–252 (1988).
- Lovinger, D. M., Wong, K. L., Murakami, K. & Routtenberg, A. *Brain Res.* **436**, 177–183 (1987).
- Malinow, R., Madison, D. V. & Tsien, R. W. *Science* **245**, 862–866 (1989).
- Reymann, K. G., Frey, U., Jork, R. & Matthies, H. *Brain Res.* **440**, 305–314 (1988).
- Sladeczek, F., Pin, J.-P., Recasens, M., Bockaert, J. & Weiss, S. *Nature* **317**, 717–719 (1985).
- Nicoletti, F. *et al. J. Neurochem.* **46**, 40–46 (1986).
- Harvey, J., Frenguelli, B. G., Sunter, D. C., Watkins, J. C. & Collingridge, G. L. *Br. J. Pharmacol.* **104**, c79 (1991).
- Kelso, S. R., Nelson, T. E. & Leonard, J. P. *J. Physiol., Lond.* **449**, 705–718 (1992).
- Aniksztejn, L., Otani, S. & Ben-Ari, Y. *Eur. J. Neurosci.* **4**, 500–505 (1992).
- Malenka, R. C. *Neuron* **6**, 53–60 (1991).
- Dumuis, A., Pin, J. P., Oomagari, K., Sebben, M. & Bockaert, J. *Nature* **347**, 182–184 (1990).
- Irving, A. J., Collingridge, G. L. & Schofield, J. G. *Cell Calcium* **13**, 293–301 (1992).
- Martin, M. R. *Neuropeptides* **4**, 45–30 (1983).
- Bramham, C. R. *Neurochem. Int.* **20**, 441–445 (1992).
- Derrick, B. E., Rodriguez, S. B., Lieberman, D. N. & Martinez, J. L. Jr. *J. Pharmacol. exp. Ther.* **263**, 725–733 (1992).
- Blanton, M. G., LoTurco, J. J. & Kreigstein, A. R. *J. Neurosci. Meth.* **30**, 203–210 (1989).
- Davies, S. N. & Collingridge, G. L. *Proc. R. Soc. B* **236**, 373–384 (1989).

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† To whom correspondence should be addressed.

Figure 2 summarizes the changes in SSC frequency and amplitude under various treatment conditions. Treatment with BDNF (50 ng ml^{-1}) produced an increase in SSC frequency similar to NT-3 (6.3 ± 1.8 times the control; s.e., $n = 14$), whereas NGF (50 ng ml^{-1}) was apparently without effect (1.6 ± 0.5 times the control; s.e., $n = 14$). The kinase inhibitor K252a inhibits the activation of the Trk receptors without affecting other tyrosine kinases, and prevents the autophosphorylation of Trk receptors as well as biological responses to NGF, BDNF and NT-3 (refs 13–15). In the presence of K252a (200 nM), the change in SSC frequency produced by addition of NT-3 was inhibited (1.5 ± 0.2 times the control; s.e., $n = 8$; Fig. 2a). In contrast, the same concentration of K252b, a related kinase inhibitor that does not inhibit the Trk receptors at this concentration¹⁶, did not prevent the NT-3-induced increase in SSC frequency (8.9 ± 2.8 times the control; s.e., $n = 8$; Fig. 2a). This result indicates that the Trk receptors, which are activated by neurotrophins in other sys-

tems^{7,17,20}, are likely to mediate the increase in synaptic activity. The change in the mean SSC amplitude was also determined after the various treatments; no significant changes were found for any of the treatment conditions (ANOVA; Fig. 2b). These *Xenopus* cultures consist of a heterogeneous population of neurons^{11,12,21}; variability in the NT-3 and BDNF effects may be accounted for by differential effects of the neurotrophins on different subpopulations of cholinergic neurons.

The effect of neurotrophins on the impulse-evoked synaptic response was also examined by recording the membrane currents of innervated myocytes in response to extracellular suprathreshold stimulation of the neuronal soma at a low frequency. A gradual increase in the mean amplitude of the evoked synaptic currents (ESCs) was observed following the addition of NT-3 (50 ng ml^{-1}) to the bath (Fig. 3a). BDNF produced a similar increase in ESC amplitude, whereas NGF was without effect. Figure 3b shows the mean ESC amplitudes for different treat-

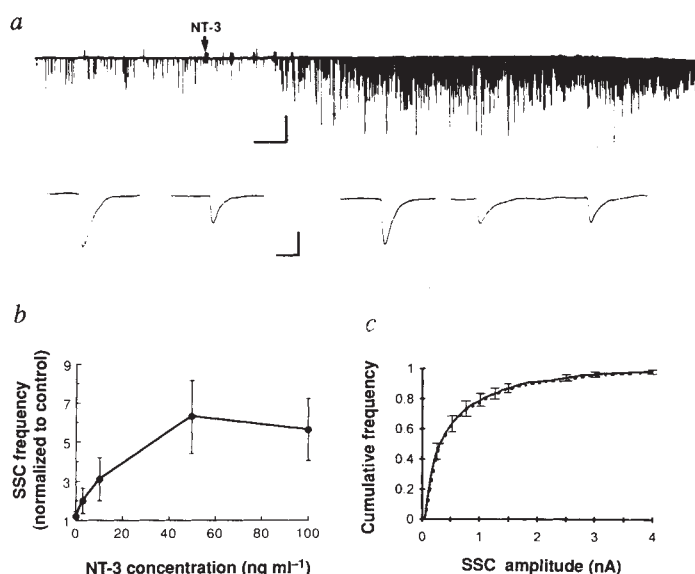
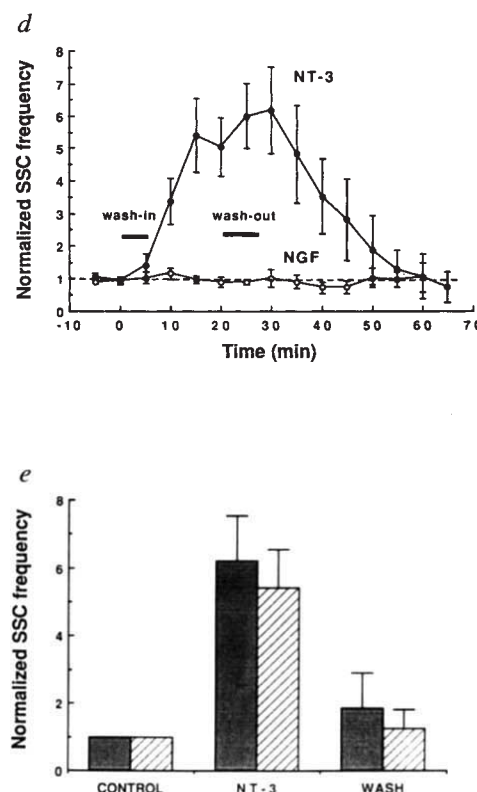


FIG. 1 Potentiation of spontaneous synaptic activity by neurotrophin-3 (NT-3) at *Xenopus* nerve-muscle synapses. **a**, Continuous trace depicts membrane current of an innervated myocyte ($V_h = -60 \text{ mV}$, filtered at 150 Hz) in a 1-day-old culture. Downward events are inward spontaneous synaptic currents (SSCs) resulting from spontaneous secretion of ACh from the presynaptic neuron^{11,12}. Arrow marks the onset of perfusion with culture medium containing 50 ng ml^{-1} NT-3. Examples of SSCs are shown by the traces below at higher time resolution (filtered at 2.5 kHz). Scales: slow trace, 500 pA and 2 min ; fast trace, 1 nA and 10 ms . **b**, The mean SSC frequency 40 min after the application of NT-3 at different concentrations was normalized for each synapse by setting the mean SSC frequency before the NT-3 addition to 1. Error bars represent s.e. The difference between the value at 50 ng ml^{-1} and 0 or 3 ng ml^{-1} is statistically significant ($P < 0.05$, ANOVA and *post-hoc* comparisons). **c**, Distribution of SSC amplitudes before and after NT-3 treatment. Events during a period $30\text{--}40 \text{ min}$ after the application of NT-3 (50 ng ml^{-1}) were analysed. The cumulative frequency refers to the proportion of total events with amplitudes smaller than a given amplitude before (dashed line) and after (solid line) the treatment. Curves represent averaged amplitude distributions from 9 synapses. For clarity, error bars (s.e.) are shown only for some points along the solid line. **d**, Changes in the SSC frequency (normalized to control frequency) with time during the addition and removal of 50 ng ml^{-1} NT-3 (upper line; mean $\pm$ s.e., $n = 6$). The NT-3-containing culture medium was perfused into the recording chamber (horizontal bar, left) and was washed out after about 15 min by perfusion with fresh culture medium (horizontal bar, right). Similar treatment with NGF-containing medium is shown by the lower line (50 ng ml^{-1} NGF; mean $\pm$ s.e., $n = 6$). **e**, Increasing the time of exposure to NT-3 from 15 min (shaded bars; same data as in **d**) to 60 min (striped bars; $n = 4$) did not significantly change the extent of SSC frequency elevation, and the effect was similarly abolished by removal of NT-3. (No differences between the 15-min and 60-min groups; Mann-Whitney U-test, $P > 0.05$.)



METHODS. Culture procedures have been described³⁴. Briefly, neural tubes and the associated myotomal muscle tissue were taken from stage 20–22 *Xenopus* embryos, allowed to dissociate in Ca^{2+} - and Mg^{2+} -free Ringer's solution, and plated on glass coverslips. At this stage of development, the neural tube has closed but neurons have not yet made synaptic contacts with the muscle cells. The culture medium consisted of 50% (vol/vol) Leibovitz medium (GIBCO), 2% fetal bovine serum (GIBCO) and 48% Ringer's solution (115 mM NaCl , 2 mM CaCl_2 , 2.5 mM KCl , 10 mM HEPES , pH 7.3). The spinal neurons survive and maintain functional synapses with muscle cells for 2–3 days under normal culture conditions. Although the survival can be promoted by chronic treatment with BDNF or NT-3, cultures used here were not so treated. Whole-cell patch-clamp recordings³⁵ were made in culture medium after 1 d of incubation at room temperature ($20\text{--}22^\circ \text{C}$). The solution inside the recording pipette contained 150 mM KCl , 1 mM NaCl , 1 mM MgCl_2 and 10 mM HEPES buffer, pH 7.2. It has been shown that 80–90% of neurons in these cultures can release ACh^{11,12}. The membrane current in all recordings was monitored by a patch-clamp amplifier (List EPC-7) and chart recordings from an oscillographic recorder (Gould RS 3200). Data were stored on a videotape recorder for later playback onto a storage oscilloscope (Tektronix 5113) and for analysis by a microcomputer.

ments, normalized to the control ESC amplitude for each synapse. Statistical analysis (ANOVA and *post-hoc* comparisons) of the ESC amplitudes 15–25 min after addition of the factor showed that synapses treated with NT-3 or BDNF produced ESCs of significantly greater amplitude than those recorded from either NGF-treated or control synapses (with no factor added). A gradual decline in the mean amplitude was observed in the latter two sets of recordings, presumably as a result of slow synaptic depression caused by the test stimulation²². In the presence of 200 mM K252a, no significant effect of NT-3 on the impulse-evoked responses was observed over a 30-min recording period ($n=4$; data not shown), suggesting that Trk receptors also mediate this effect. The increase in ESC amplitude produced by NT-3 or BDNF is likely to be the result of an increased number of quanta released in response to the action potential. Increased post-synaptic receptor sensitivity or increased amount of transmitter per quantum are unlikely mechanisms, because neither the SSC amplitude distribution nor the mean SSC amplitude changed after addition of the neurotrophins (Figs 1c and 2b). Taken together, our observations of increased SSC frequency and ESC amplitude are consistent with a neurotrophin-induced increase either in the probability of quantal release or in the number of available quanta.

Studies of neurotrophin expression in the adult mammal have shown that peripheral target tissues of spinal neurons express NT-3 and BDNF^{3,4,23}, raising the possibility that NT-3 and/or BDNF may act as target-derived trophic factors for spinal

neurons. It is possible that muscle cells in our culture could provide some neurotrophin that acts on the co-cultured neurons, although under our usual low-density culture conditions the amount of putative factors produced by the muscle cells appears to be insufficient for maintaining long-term neuronal survival. We have observed decreased neuronal death and increased neurite outgrowth when these cultured *Xenopus* neurons are treated with either BDNF or NT-3, but not NGF (Z. Zheng, A.M.L. and M.P., unpublished data). In this context, it is interesting to note that BDNF and NT-3, but not NGF, upregulate the cholinergic phenotype of purified rat embryonic motor neurons *in vitro*²⁴. In addition, it has been shown that *in vivo* BDNF and NT-3, but not NGF, can be retrogradely transported by adult rat motor neurons following injection of labelled neurotrophins into the sciatic nerve²⁵, and that BDNF decreases naturally occurring motor neuron cell death²⁶ as well as motor neuron cell death after injury^{26–28}. Our findings suggest that neurotrophins, derived from peripheral target tissues, central neurons, or the presynaptic neuron itself, may modulate the level of synaptic activity at developing synapses. Neuronal activity at developing synapses is crucial in synapse maturation

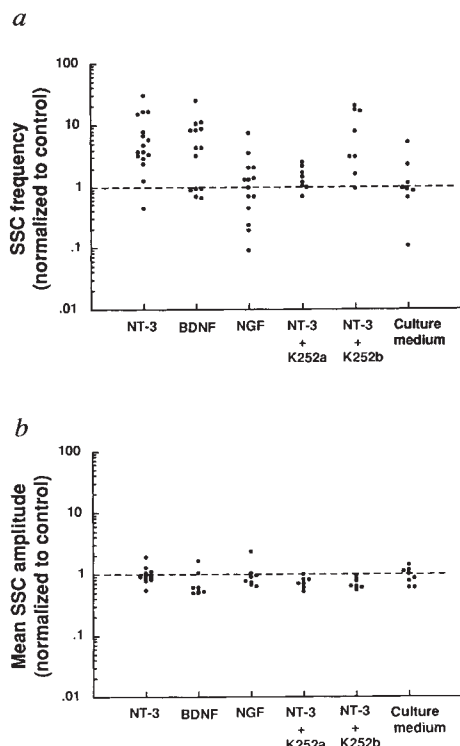


FIG. 2 Effects of neurotrophins and kinase inhibitors on *a*, SSC frequency, and *b*, amplitude. All neurotrophins were applied at a concentration of 50 ng ml⁻¹. Each point represents the results of one experiment. The SSC frequency and mean SSC amplitude were determined 25–40 min after bath application of NT-3, BDNF, NGF or culture medium, and normalized to the frequency and amplitude during the control period of that experiment. In some experiments cells were pretreated with 200 nM K252a (NT-3 + K252a) or with 200 nM K252b (NT-3 + K252b) for 1 h before NT-3 application. Statistical analysis (ANOVA and *post-hoc* comparisons) indicates that SSC frequencies were significantly increased ($P < 0.05$) by NT-3, BDNF, and NT-3 + K252b, as compared to culture medium controls, NGF or NT-3 + K252a. There were no significant differences in mean SSC amplitudes among all groups. Lateral displacement of the data points is for clarity of presentation only.

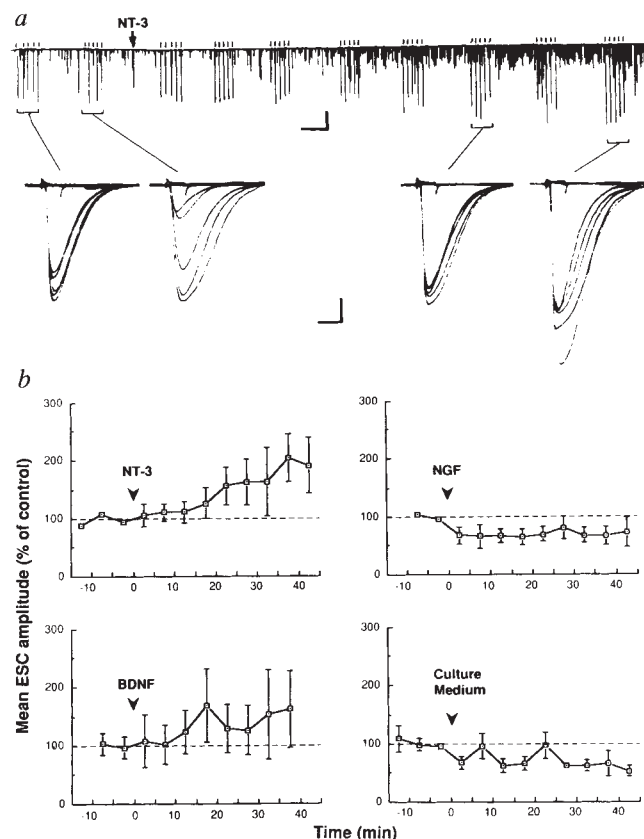


FIG. 3 Potentiation of evoked synaptic currents (ESCs) by NT-3 and BDNF. *a*, Continuous trace depicts the membrane current recorded from an innervated myocyte under voltage-clamp ($V_h = -60$ mV, filtered at 150 Hz, inward current downward). ESCs were elicited at low frequency at times marked by the small vertical lines. Arrow marks the time of bath application of NT-3 (50 ng ml⁻¹). Insets below are ESCs at higher time resolution (filtered at 2.5 kHz) for the recording periods indicated. The 7-ms artefacts on the base line were due to tests of seal resistance. Scales: slow trace, 1.5 nA and 2 min; fast traces, 1 nA and 10 ms. *b*, Changes in ESC amplitudes after treatment with NT-3 ($n=7$), BDNF ($n=6$) or NGF ($n=6$), added at the time marked by the arrows. Also shown are data from control experiments in which medium containing no neurotrophin was added to the culture ($n=5$). Mean ESC amplitudes at different times were normalized for each synapse by setting the mean amplitude before neurotrophin application to 100%. Error bars represent s.e.

and competition²² as well as in the differentiation of postsynaptic properties²⁹. It is interesting to note that the levels of neurotrophin messenger RNAs in hippocampal neurons can be regulated by electrical activity^{1,30-33}. Thus a reciprocal regulation between neurotrophin expression and synaptic activity may operate to modulate synaptic efficacy.

In conclusion, our results provide the first evidence, to our knowledge, that neurotrophins can rapidly enhance the activity and efficacy of developing neuromuscular synapses. This finding supports the notion that neurotrophic factors are directly involved in regulating the formation and function of neuromuscular synapses during development. It remains to be determined whether such synaptic regulation also occurs at other synapses and whether the immediate effects on synaptic activity lead to long-term structural or functional changes at the synapse. □

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1. Thoenen, H. *Trends Neurosci.* **14**, 165-170 (1991).
2. Lo, D. C. *Curr. Opin. Neurobiol.* **2**, 336-340 (1992).
3. Hohn, A., Leibrock, J., Bailey, K. & Barde, Y. A. *Nature* **344**, 339-341 (1990).
4. Maisonnier, P. C. *et al. Science* **247**, 1446-1451 (1990).
5. Leibrock, J. *et al. Nature* **341**, 149-152 (1989).
6. Levi-Montalcini, R. *EMBO J.* **6**, 1145-1154 (1987).

7. Chao, M. V. *Neuron* **9**, 583-593 (1992).
8. Alderson, R. F., Alterman, A. L., Barde, Y.-A. & Lindsay, R. M. *Neuron* **5**, 297-306 (1990).
9. Hyman, C. *et al. Nature* **350**, 230-232 (1991).
10. Ip, N. Y. *et al. Neuron* **10**, 137-149 (1993).
11. Evers, J., Laser, M., Sun, Y., Xie, Z. & Poo, M.-m. *J. Neurosci.* **9**, 1523-1539 (1989).
12. Xie, Z.-P. & Poo, M.-m. *Proc. natn. Acad. Sci. U.S.A.* **83**, 7069-7073 (1986).
13. Berg, M. M., Sternberg, D. W., Parada, L. F. & Chao, M. V. *J. Biol. Chem.* **267**, 13-16 (1992).
14. Tapley, P., Lamballe, F. & Barbacid, M. *Oncogene* **7**, 371-381 (1992).
15. Nye, S. H. *et al. Molec. Biol. Cell* **3**, 677-686 (1992).
16. Knusel, B. & Hefti, F. *J. Neurochem.* **57**, 955-962 (1991).
17. Squinto, S. P. *et al. Cell* **65**, 885 (1991).
18. Soppet, D. *et al. Cell* **65**, 895 (1991).
19. Lamballe, F., Klein, R. & Barbacid, M. *Cell* **66**, 967-979 (1991).
20. Cordon-Cardo, C. *et al. Cell* **66**, 173-183 (1991).
21. Bixby, J. L. & Spitzer, N. C. *J. Physiol., Lond.* **353**, 143-155 (1984).
22. Lo, Y.-J. & Poo, M.-m. *Science* **254**, 1019-1022 (1991).
23. Maisonnier, P. C. *et al. Neuron* **5**, 501-509 (1990).
24. Wong, V., Arriaga, R., Ip, N. Y. & Lindsay, R. M. *Eur. J. Neurosci.* **5**, 466-474 (1993).
25. DiStefano, P. S. *et al. Neuron* **8**, 983-993 (1992).
26. Oppenheim, R., Qin-Weim, Y., Prevette, D. & Yan, Q. *Nature* **360**, 755-757 (1992).
27. Yan, Q., Elliott, J. & Snider, W. D. *Nature* **360**, 753-755 (1992).
28. Sendtner, M., Holtmann, B., Kolbeck, R., Thoenen, H. & Barde, Y.-A. *Nature* **360**, 757-759 (1992).
29. Kidokoro, Y. & Saito, M. *Proc. natn. Acad. Sci. U.S.A.* **85**, 1978-1982 (1988).
30. Zafra, F., Hengeler, B., Leibrock, J., Thoenen, H. & Lindholm, D. *EMBO J.* **9**, 3545-3550 (1990).
31. Isackson, P. J., Huntsman, M. M., Murray, K. D. & Gall, C. M. *Neuron* **6**, 937-948 (1991).
32. Lu, B., Yokoyama, M., Dreyfus, C. F. & Black, I. B. *Proc. natn. Acad. Sci. U.S.A.* **88**, 6289-6292 (1991).
33. Patterson, S. L., Grover, L. M., Schwartzkroin, P. A. & Bothwell, M. *Neuron* **9**, 1081-1088 (1992).
34. Tabti, N. & Poo, M.-m. in *Culturing Nerve Cells* (eds Banker, G. & Goslin, K.) 137-153 (MIT, Cambridge, MA, 1991).
35. Hamill, O. P., Marty, A., Neher, E., Sakmann, B. & Sigworth, F. J. *Pfluegers Arch.* **391**, 85-100 (1981).

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Tyrosine kinase-dependent selection of transmitter responses induced by neuronal contact

Stefano Catarsi & Pierre Drapeau

McGill University Centre for Research in Neuroscience and Montreal General Hospital Research Institute, 1650 Cedar Avenue, Montreal, Quebec, Canada H3G 1A4

TRANSMITTER receptors are localized to discrete cellular sites¹ such that only those responses appropriate for a particular pattern of inputs are activated²⁻⁴. How neurons select between synaptic and extrasynaptic responses during development is not understood. We have investigated how contact during synapse formation between identified leech neurons⁵⁻⁷ selectively suppresses the modulation of extrasynaptic channels by protein kinase C⁸⁻¹⁰. A microelectrode with an isolated membrane patch containing channels from an uninnervated target neuron was 'crammed'¹¹ into a similar cell contacted by a presynaptic partner. We report here that within a few minutes, the crammed channels were rendered insensitive to activation of protein kinase C, demonstrating the action of a cytoplasmic signal. Treatment of the neurons with selective inhibitors of tyrosine kinases¹², which are signalling molecules during normal and oncogenic cellular differentiation^{13,14}, prevented the loss of channel modulation. Thus, tyrosine kinases mediate early functional changes during specific synapse formation that are induced by neuronal contact.

When inhibitory synapses reform between serotonergic Retzius (R) and pressure (P) neurons of the leech in culture^{5,6}, the excitatory extrasynaptic response to serotonin is eliminated at sites of contact⁷. Contact specifically with the R cell⁸ renders cation channels in the P cell insensitive to serotonin activation of the second messenger protein kinase C (PKC)^{9,10}. To investigate how contact suppresses channel modulation, a microelectrode with cation channels from an uninnervated P cell was crammed into the same P cell or into a P cell contacted by an R cell. Cation channels were identified in P-cell-attached patch clamp recordings (Fig. 1, left) by their characteristic properties⁹. The patches were isolated in the inside-out configuration¹⁵ by

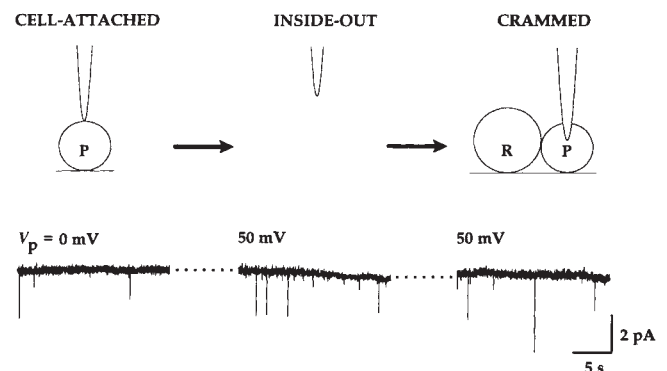
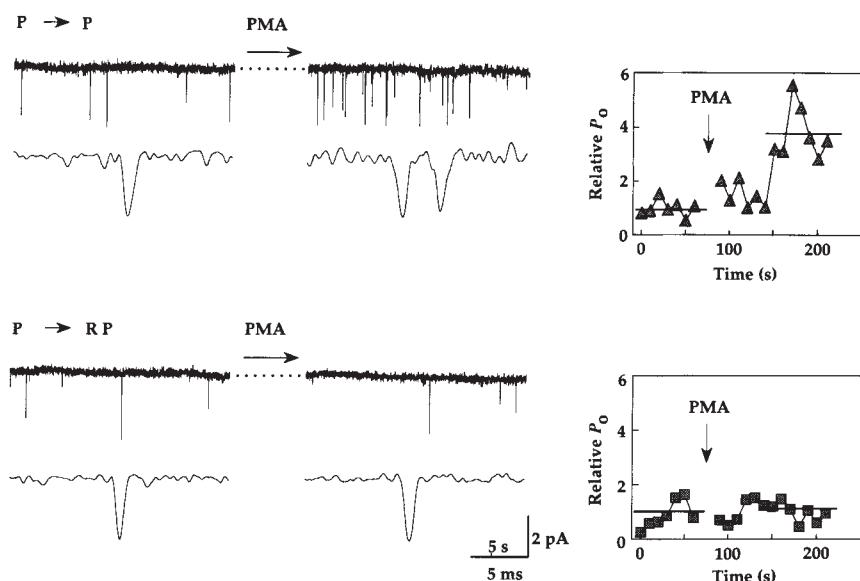


FIG. 1 Patch cramming of cation channels in cultured P cells. Top, Steps involved in patch cramming, for which sample recordings from one experiment are shown at the bottom. The pipette potential, V_p (opposite to the potential applied across the membrane patches) is indicated for each recording. Left, Cation channels are easily identified in P-cell-attached patch clamp recordings by their characteristic properties⁹, the most obvious being rare, brief inward current jumps at the resting potential (~ -50 mV). Middle, In inside-out patches, the channels are seen only on application of a pipette potential. Right, The channels are still observed several minutes after cramming of the pipette well into the P cell. The amplitude of single channel current jumps was usually maintained, consistent with a reduction of the resting potential on insertion of the blunt patch pipettes.

METHODS. R and P cells were isolated from the nervous system of the leech *Hirudo medicinalis* and cultured singly or as pairs for 3-10 days as described previously²⁹. Inside-out patches were excised after gigaohm seal formation and recorded from in a low (0.1 μ M) calcium solution¹⁰ containing 155 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 1.5 mM EGTA, 10 mM glucose and 10 mM HEPES (pH 7.4). Pipettes with excised patches were then approached to the P cells until they were seen to dimple or move and were inserted a further 10 μ m using a motorized manipulator with calibrated steps. After recording basal channel activity for a few minutes, a final concentration of 0.5 μ M PMA (diluted 2,000-fold from a stock solution dissolved in dimethylsulphoxide (DMSO); LC Services, Woburn, MA) was added. The records were filtered at 1 kHz (-30 dB), digitized at 5 kHz during transfer to storage tape and then redigitized at 75 Hz (in order to show 20 s segments). Because the mean open times were ~ 1 ms, brief events appeared small^{9,10}.

FIG. 2 Effect of R-cell contact on cation channel modulation in crammed patches from single P cells. Top, Cation channel activity in a patch excised from a single P cell and crammed back into it (left) was increased on addition of PMA at a final concentration of $0.5 \mu\text{M}$ (middle), as summarized in the running plot of the probability of channel opening (P_o ; right). Bottom, In contrast, channel activity in a patch excised from a single P cell and crammed into another P cell contacted by an R cell (left; same experiment shown in Fig. 1) was unaffected by PMA application (middle), as summarized in the running plot of P_o (right).

METHODS. The records were filtered as described in Fig. 1 and 20-s segments are shown for comparison. Channel openings are also shown on a 1,000-fold expanded scale for recordings redigitized at 10 kHz, in which discrete openings are fully resolved. As shown in the running plots of P_o , activation by PMA, when observed, reached a maximal rate within 1 min after application. The activity level (relative to that observed before PMA addition) is indicated for the data points 1 min after PMA addition. The average levels of activity were top, control: $P_o=0.0010$, amplitude= 1.9 pA ($1.61 \pm 0.27 \text{ pA}$, $n=9$), open time= 1.2 ms ($0.84 \pm 0.09 \text{ ms}$, $n=9$); top, +PMA: $P_o=0.0039$, amplitude= 2.0 pA ($1.82 \pm 0.20 \text{ pA}$, $n=9$), open time= 1.0 ms (0.83 ± 0.09 , $n=9$); bottom, control: $P_o=0.0026$, amplitude= 2.0 pA



($1.69 \pm 0.15 \text{ pA}$, $n=11$), open time= 1.1 ms ($0.84 \pm 0.15 \text{ ms}$, $n=11$); and bottom, +PMA: $P_o=0.0029$, amplitude= 1.9 pA (1.82 ± 0.17 , $n=11$) and open time= 1.3 ms ($0.96 \pm 0.13 \text{ ms}$, $n=11$).

excision in a low calcium ($0.1 \mu\text{M}$) solution¹⁰ and channels were detected on application of a potential in the pipette (Fig. 1, middle). The pipette was then crammed $\sim 10 \mu\text{m}$ into the cytoplasm of the P-cell soma (Fig. 1, right).

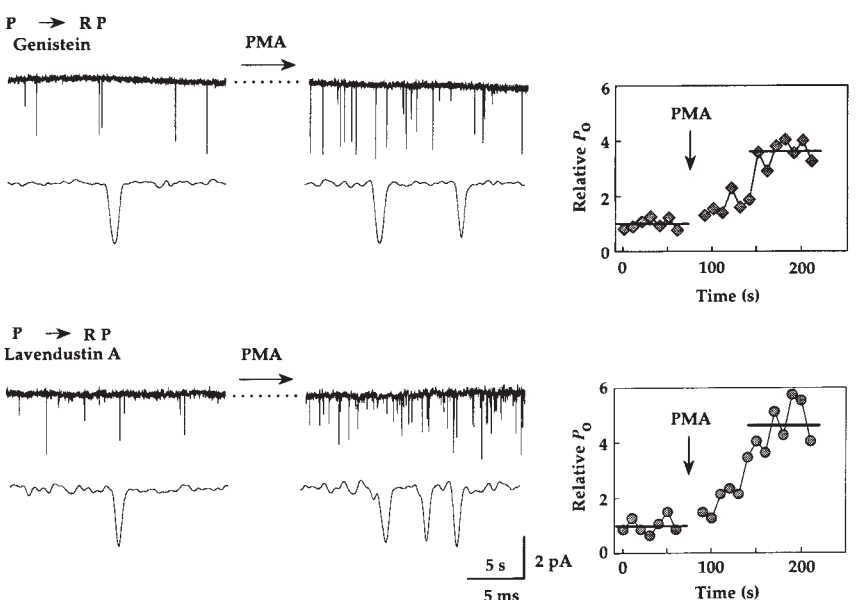
After cramping the patches, the probability of channel opening was measured by recording several hundred spontaneous openings. Subsequent addition of phorbol myristate acetate (PMA), which activates PKC¹⁶, increased cation channel activity nearly fourfold in patches crammed into single P cells (Fig. 2, top and Fig. 4). This stimulation was weaker than the 10-fold increase in activity observed with intact, P-cell-attached recordings on application of PMA⁹ or with isolated, inside-out patches on application of purified PKC¹⁰, probably because less PKC

was available to patches crammed into the cytoplasm. As expected from our previous work⁶⁻¹⁰, patches excised from P cells contacted by R cells and crammed back into the contacted P cells were far less sensitive to PMA (Fig. 4).

To test the timing and nature of the effects of contact on channel modulation, patches were isolated from single P cells and crammed into other, neighbouring P cells contacted by R cells. Because openings are rare, channel activity was recorded for 2 to 5 minutes. Addition of PMA did not affect channel activity (Fig. 2, bottom and Fig. 4). This result suggests that a cytoplasmic signal in contacted P cells rapidly rendered the crammed channels from single P cells insensitive to modulation by subsequent activation of PKC. To determine whether this

FIG. 3 Effect of tyrosine kinase inhibitors on cation channel modulation in patches from single P cells crammed into paired P cells. Top, Cation channel activity for cells treated with genistein (left) was increased on addition of PMA at a final concentration of $0.5 \mu\text{M}$ (middle), as summarized in the running plot of the probability of channel opening (P_o ; right). Bottom, Similarly, channel activity for cells treated with Lavendustin A (left) was unaffected by PMA application (middle), as summarized in the running plot of P_o (right).

METHODS. The genistein and Lavendustin A treatments consisted of exposing P cells to either compound ($20 \mu\text{M}$ genistein diluted 2,000-fold from a stock solution in DMSO or $10 \mu\text{M}$ Lavendustin A diluted 5,000-fold from a stock solution dissolved in DMSO; LC Services, Woburn, MA) for 2-4 h before pairing, plating single P cells and P cells paired with R cells in culture medium supplemented with either compound for 3 days and recording in low calcium solution, always in the presence of either compound. The records were obtained as described in Fig. 2 and the average levels of activity were top, control: $P_o=0.0036$, amplitude= 3.0 pA ($2.01 \pm 0.25 \text{ pA}$, $n=8$), open time= 1.9 ms ($1.33 \pm 0.35 \text{ ms}$, $n=8$); top, +PMA: $P_o=0.0138$, amplitude= 2.3 pA ($2.06 \pm 0.31 \text{ pA}$, $n=8$), open time= 1.9 ms ($1.23 \pm 0.15 \text{ ms}$, $n=8$); bottom, control: $P_o=0.0047$, amplitude= 1.9 pA ($1.71 \pm 0.19 \text{ pA}$, $n=8$), open time= 0.8 ms



($1.10 \pm 0.22 \text{ ms}$, $n=8$); and bottom, +PMA: $P_o=0.0217$, amplitude= 1.8 pA (1.78 ± 0.19 , $n=8$) and open time= 0.8 ms ($1.28 \pm 0.26 \text{ ms}$, $n=8$).

process was reversible, patches were either (1) isolated from contacted P cells or (2) isolated from single P cells, crammed into contacted P cells and shown to be rendered insensitive to PMA; the patches were then crammed into single P cells. No significant increase in activity was found on average (Fig. 4), although one of the eight patches showed a recovery of sensitivity to PMA (3.5-fold increase compared to less than 2-fold for the 7 other experiments). The loss of channel sensitivity to PKC was maintained for at least half an hour, the longest period that the recordings lasted, demonstrating that channel modulation was stably suppressed.

The differentiation of *Drosophila* photoreceptors¹³, assembly of growth cone tubulin¹⁷ and clustering of muscle acetylcholine receptors (AChRs) during synaptogenesis^{18,19} that are induced by cell contact appear to depend on tyrosine kinase activity. To determine if tyrosine kinases participate in the loss of cation channel modulation, neurons were treated starting 2–4 hours before pairing and throughout the several days of R–P pairing with the selective tyrosine kinase inhibitor genistein or, as a control, with inactive genistin¹². Treatment with genistein (Fig. 3, top; Fig. 4) but not genistin (Fig. 4) prevented the loss of channel modulation when patches from single P cells were crammed into other, contacted P cells because PMA was still able to activate the channels. Because Lavendustin A has recently been described to be more selective than genistein as an inhibitor of tyrosine kinases compared to other kinases¹², we also incubated cells with Lavendustin A or, as a control, the inactive B form. Treatment with Lavendustin A (Fig. 3, bottom; Fig. 4) but not Lavendustin B (Fig. 4) also prevented the loss of channel modulation.

Two possibilities could account for channel stimulation by PMA in the presence of the tyrosine kinase inhibitors: the inhibitors either stimulated the PKC cascade directly or blocked

an event triggered by cell contact that normally suppresses channel modulation by PKC. Cell pairs remained adhered following drug treatment, as observed by the inability to separate the cells when they were displaced from the culture dish. To test for a stimulatory effect of the drugs on the PKC cascade, we incubated the cells (after pairing) for 0.5–3 hours with Lavendustin A before starting the recordings. We found that Lavendustin A failed to block the loss of sensitivity to PMA (0.92 ± 0.12 -fold effect of PMA, $n = 6$). This result suggests that the suppression of channel modulation was induced by cell contact and required tyrosine kinase activity.

It is interesting to compare these results with the detailed information obtained from studies of the cellular and molecular events during synapse formation at the neuromuscular junction. At this synapse, neuronal contact triggers the clustering of AChRs in the muscle fibre^{20,21} and induces a functional change in AChR properties^{22,23}. The molecular basis for the functional modification is the switching of AChR subunit composition²⁴ which is controlled locally by subjunctional nuclei in the muscle fibre²⁵. In contrast to the transcriptional control of AChR structure and function in muscle, our results suggest that a tyrosine kinase-dependent modification of extrasynaptic P-cell channels on contact specifically with the R cell underlies the stable loss of channel modulation and implicate this important class of signalling molecules in neuronal synapse formation. Distinct from the specialized function of subsynaptic nuclei in muscle cells, the subsynaptic regions of neurons along dendritic shafts are at considerable distances from the nucleus. Therefore, local interactions at sites of neuronal contact could play an important early role during synapse formation.

The selection of transmitter responses at sites of contact is the first functional event detected during formation of the synapse between R and P cells⁷. Accordingly, the subsequent differentiation of growth cones into functional nerve endings would be expected to result in activation of only the correct, inhibitory responses²⁶. Competition between synapses determines the final pattern of connections²⁷ and coincident pre- and postsynaptic activity stabilizes excitatory synapses²⁸. The early contact-induced selection of postsynaptic responses would confer a competitive advantage to neurons that may be particularly important during the formation of inhibitory synapses for which activity patterns may be less determinant. □

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- Hille, B. *Ionic Channels of Excitable Membranes* (Sinauer, Sunderland, MA, 1992).
- Gerschlager, H. M. *Physiol. Rev.* **53**, 1–119 (1973).
- Nicoll, R. A. *Science* **241**, 545–551 (1988).
- Nicoll, R. A., Malenka, R. C. & Kauer, J. A. *Physiol. Rev.* **70**, 513–565 (1991).
- Fuchs, P., Nicholls, J. G. & Ready, D. F. *J. Physiol., Lond.* **316**, 203–233 (1981).
- Drapeau, P. & Sanchez-Armass, S. *J. Neurosci.* **8**, 4718–4727 (1988).
- Drapeau, P., Melnyshyn, E. & Sanchez-Armass, S. *J. Neurosci.* **9**, 2502–2508 (1989).
- Merz, D. & Drapeau, P. *Proc. R. Soc. B248*, 129–133 (1992).
- Drapeau, P. *Neuron* **4**, 875–882 (1990).
- Catarsi, S. & Drapeau, P. *Neuron* **8**, 275–281 (1992).
- Kramer, R. H. *Neuron* **2**, 335–341 (1990).
- O'Dell, T. J., Kandel, E. R. & Grant, S. G. N. *Nature* **353**, 558–560 (1991).
- Greenwald, I. & Rubin, G. M. *Cell* **68**, 271–281 (1992).
- Cantley, L. C. et al. *Cell* **64**, 281–302 (1991).
- Hamill, O. P. et al. *Pflügers Arch.* **391**, 85–100 (1981).
- Nishizuka, Y. *Nature* **334**, 661–665 (1988).
- Atashi, J. R. et al. *Neuron* **8**, 831–842 (1992).
- Qu, Z., Moritz, E. & Hagan, R. L. *Neuron* **2**, 367–378 (1990).
- Wallace, B. G., Qu, Z. & Hagan, R. L. *Neuron* **6**, 869–878 (1991).
- Cohen, M. W., Rodriguez-Marín, E. & Wilson, E. M. *J. Neurosci.* **7**, 2849–2861 (1987).
- Bloch, R. J. & Pimplin, D. W. *Am. J. Physiol.* **254**, C345–C364 (1988).
- Katz, B. & Miledi, R. *J. Physiol., Lond.* **224**, 665–699 (1972).
- Neher, E. & Sakmann, B. *J. Physiol., Lond.* **258**, 705–729 (1976).
- Mishina, M. et al. *Nature* **321**, 406–411 (1986).
- Witzemann, V., Brenner, H.-R. & Sakmann, B. *J. Cell Biol.* **114**, 125–141 (1991).
- Drapeau, P. & Sanchez-Armass, S. *J. Neurobiol.* **20**, 312–325 (1989).
- Purves, D. & Lichtman, J. W. *Principles of Neural Development* (Sinauer, Sunderland, MA, 1985).
- Shtatz, C. J. *Neuron* **5**, 745–756 (1990).
- Dietzel, I. D., Drapeau, P. & Nicholls, J. G. *J. Physiol., Lond.* **372**, 191–205 (1986).

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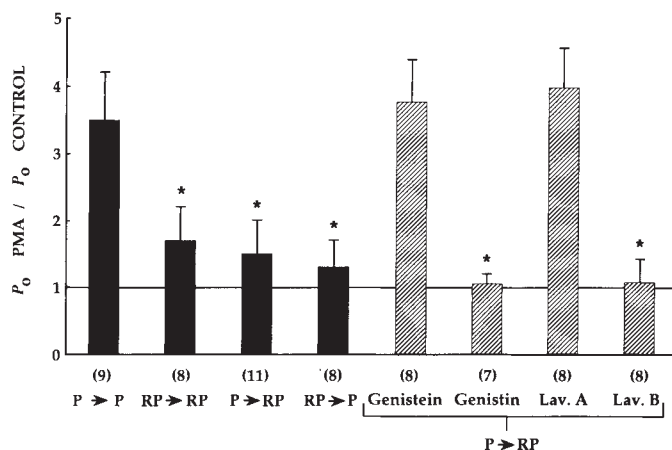


FIG. 4 Summary of patch clamp results with single and contacted P cells. The histogram shows the ratio of the channel open probability (P_o) measured for 2–5 min in the presence of PMA (after waiting at least 1 min after application to allow maximal activation) relative to P_o before PMA addition for (filled bars) patches excised from and crammed into single P cells (P → P) or contacted P cells (RP → RP) or removed from single P cells and crammed into other, contacted P cells (P → RP) or removed from contacted P cells and crammed into single P cells (RP → P); half (4) of the latter patches were isolated from single P cells, crammed into contacted P cells and shown to be unaffected by PMA before being crammed into single P cells. As indicated for the cross-hatched bars at the right, some cells were treated with genistein, genistin (Extrasynthèse, Orsay, France), Lavendustin A or B (as described in the legend to Fig. 3) before cramping patches from single P cells into other, contacted P cells. The number of patches recorded from is shown in parentheses below each group of measurements and the error bars are s.e.m. The asterisks indicate results that were significantly different ($P < 0.05$ by the two-tailed Student's t -test) from those with patches excised from and crammed into single P cells (P → P). The individual P values for each group are (starting with RP → RP) 0.036, 0.017, 0.010, 0.848, 0.005, 0.648, 0.005.

Calcium-independent potentiation of insulin release by cyclic AMP in single β -cells

Carina Ämmälä, Frances M. Ashcroft* & Patrik Rorsman†

Department of Medical Biophysics, Medicinaregatan 11, S-413 90 Göteborg, Sweden

* Permanent address: University Laboratory of Physiology, Parks Road, Oxford OX1 3PT, UK.
† To whom correspondence should be addressed.

How does cyclic AMP potentiate insulin secretion from pancreatic islet β -cells? This question is fundamental to understanding how hormones such as glucagon, which elevates cAMP¹, stimulate insulin secretion and so contribute to the normal secretory response of the islet^{2,3}. It is well established that a rise in the cytoplasmic Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) is essential for insulin secretion⁴ and therefore cAMP has been proposed to act by elevating $[\text{Ca}^{2+}]_i$. But studies on permeabilized β -cells indicate that cAMP increases insulin release even when $[\text{Ca}^{2+}]_i$ is held constant^{5,6}. We have used microfluorimetry and the patch-clamp technique to measure changes simultaneously in Ca^{2+} currents, $[\text{Ca}^{2+}]_i$ and exocytosis⁷⁻⁹ in a single β -cell in response to cAMP. We show here that cAMP, through activation of protein kinase A, increases Ca^{2+} -influx through voltage-dependent L-type Ca^{2+} channels, thereby elevating $[\text{Ca}^{2+}]_i$ and accelerating exocytosis. More importantly, cAMP also promotes insulin release by a direct interaction with the secretory machinery, which accounts for as much as 80% of its effect.

Figure 1a illustrates the whole-cell Ca^{2+} current and cell capacitance measured in an intact β -cell¹⁰. Under control conditions (left), a 500 ms depolarization to 0 mV evokes an integrated Ca^{2+} -current of 12 pC (picocoulomb) and a step capacitance increase of 80 fF (femtofarad), corresponding to the simultaneous discharge of 40–50 secretory granules²³. After addition of 1 mM 8-bromo cAMP (8-Br-cAMP; Fig. 1a, middle), a membrane-permeant cAMP analogue, the same depolarization produced an integrated Ca^{2+} current of 15 pC (an increase of 30%) but a very much larger capacitance change of 220 fF (an increase of 175%). These effects were fully reversed on removal of 8-Br-cAMP. On average (Fig. 1b), agents that elevate the intracellular cAMP concentration reversibly increased the Ca^{2+} current by $45 \pm 11\%$ ($n = 10$) and potentiated exocytosis by $360 \pm 100\%$ ($n = 13$). The principal action of cAMP on the Ca^{2+} current is to slow inactivation rather than increasing the magnitude of the current¹¹, which may explain many of the effects of cAMP-increasing agents on β -cell electrical activity^{12,13}.

The effects of cAMP both on the Ca^{2+} current and on exocytosis are probably mediated by activation of protein kinase

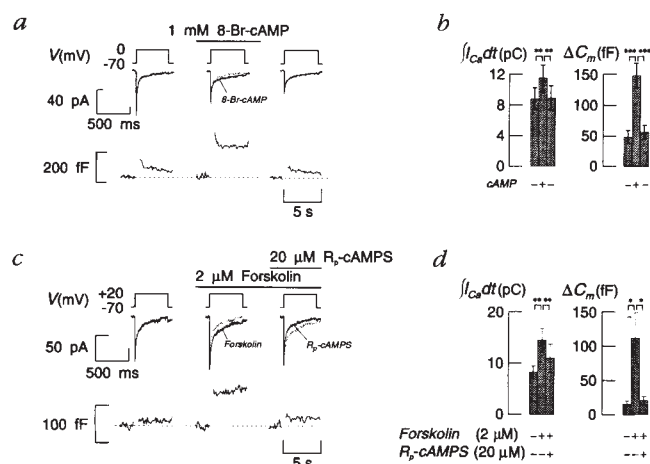


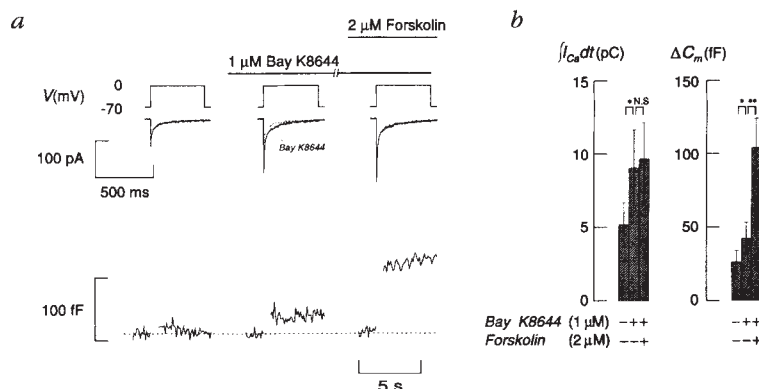
FIG. 1 *a*, Ca^{2+} currents (top) and changes in capacitance (bottom) evoked by 500-ms depolarizations before, 6 min after addition of 1 mM Br-cAMP and 8 min after its removal. *b*, Effects of cAMP on integrated Ca^{2+} current ($\int I_{\text{Ca}} dt$) and the increase in cell capacitance (ΔC_m). *c*, Ca^{2+} currents and changes in cell capacitance before, 3 min after addition of 2 μM forskolin and 7 min after adding 20 μM Rp-cAMPS. *d*, Effects of forskolin and Rp-cAMPS on the integrated Ca^{2+} current ($\int I_{\text{Ca}} dt$) and the increase in cell capacitance (ΔC_m). In *a* and *c*, Ca^{2+} currents observed under the earlier experimental condition have been superimposed to aid comparison of the current responses. The dashed horizontal lines indicate the prestimulatory capacitance level. Values in *b* and *d* are means $\pm$ s.e.m. of 4–13 experiments. * $P < 0.05$; ** $P < 0.02$; and *** $P < 0.001$.

METHODS. Whole-cell currents elicited by depolarizations to 0 mV from individual tissue-cultured mouse β -cells²³ using the perforated-patch whole-cell configuration¹⁰. Exocytosis was monitored as changes in membrane capacitance^{7-9,23}. The extracellular medium consisted of (in mM) 118 NaCl, 20 TEA-Cl, 5.6 KCl, 1.2 MgCl_2 , 2.6 CaCl_2 , 5 glucose and 5 HEPES (pH 7.4 with NaOH). Agents that raise intracellular cAMP, that is db-cAMP (2 mM), 8-Br-cAMP (1 mM) and forskolin (2 μM , dissolved in DMSO; final concentration of DMSO: 0.04%), were added as indicated in the text and the figures. In a few experiments 20 μM Rp-cAMPS, an inhibitor of cAMP-dependent protein kinase¹¹, was added to the external solution. The pipettes were filled with 76 mM Cs_2SO_4 , 10 mM NaCl, 10 mM KCl, 1 mM MgCl_2 and 5 mM HEPES (pH = 7.35). Electrical contact was established by the addition of amphotericin B (final concentration: 0.24 mg ml^{-1}) to the pipette solution. All experiments were done at $+32$ – 34 °C. Data are presented as mean values $\pm$ s.e.m. and statistical evidence evaluated using Student's *t*-test.

A as they are reversed by Rp-cAMPS, a specific inhibitor of this enzyme¹⁴. Figure 1c shows that the adenylate cyclase activator forskolin (2 μM) produced a $71 \pm 6\%$ ($n = 5$) increase in the integrated Ca^{2+} current and a $580 \pm 178\%$ ($n = 6$) potentiation of exocytosis, both of which were reversed by 20 μM Rp-cAMPS.

To determine how much of the observed potentiation of exocytosis can be explained by enhancement of the Ca^{2+} current we used the L-type Ca^{2+} -channel agonist BAY K 8644 (ref. 15).

FIG. 2 *a*, Ca^{2+} currents (top) and changes in cell capacitance (bottom) evoked by 500-ms depolarizations to 0 mV under control conditions. 2 min after addition of 1 μM BAY K 8644 and 3 min after the addition of 2 μM forskolin in the continued presence of BAY K 8644. In the middle panel, the Ca^{2+} current observed under control conditions (dotted) has been superimposed on that recorded in the presence of BAY K 8644. The dashed horizontal lines in the lower traces indicate the prestimulatory capacitance level. *b*, Effects of BAY K 8644 and forskolin on the integrated Ca^{2+} current ($\int I_{\text{Ca}} dt$) and the increase in cell capacitance (ΔC_m). Values are means $\pm$ s.e.m. of 4–6 experiments. * $P < 0.05$; ** $P < 0.01$. BAY K 8644 and forskolin were dissolved in DMSO. The final concentration of DMSO was 0.05%. Methods are as for Fig. 1.



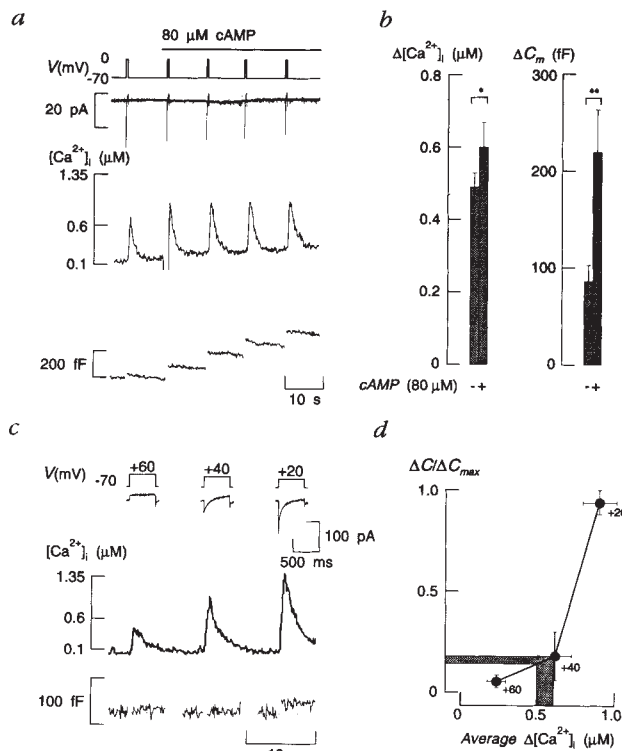


FIG. 3 *a*, Simultaneous recordings of current (top), [Ca²⁺]_i (middle) and capacitance (bottom). Five 500-ms depolarizations to 0 mV were applied at 10-s intervals. Cyclic AMP was applied just before the second depolarization. *b*, Histograms showing the effects of cAMP on the [Ca²⁺]_i transients (Δ[Ca²⁺]_i) and capacitance increases (ΔC_m). Means ± s.e.m. of seven experiments. **P* < 0.05; ***P* < 0.01. *c*, As in *a* but depolarizations went to +60 mV, +40 mV and +20 mV; that is, at the same (maximal) activation of the Ca²⁺ current²⁰. The intracellular medium lacked cAMP. *d*, [Ca²⁺]_i-dependence of exocytosis in the absence of cAMP. The capacitance change is plotted against peak [Ca²⁺]_i. Mean values ± s.e.m. of the experiment in *c* and another seven experiments. The capacitance values have been normalized to the maximum response, which was observed at either 0 mV or +20 mV. Unity corresponds to 46 ± 10 fF (*n* = 8). The shaded area indicates the potentiation of exocytosis expected from the cAMP-evoked increase in [Ca²⁺]_i shown in *b*.

METHODS. Experiments were done using the standard whole-cell configuration. The intracellular solution consisted of 125 mM Cs-glutamate, 10 mM CsCl, 10 mM NaCl, 1 mM MgCl₂, 3 mM Mg-ATP, 5 mM HEPES (pH 7.15 with CsOH) and 10 μM EGTA. Cyclic AMP was applied by photorelease from 100 μM of the caged precursor (Molecular Probes) as previously described²¹. The break in the [Ca²⁺]_i record (*a*, middle) is due to the light flash used for photolysis of the caged compound. [Ca²⁺]_i was estimated by dual emission spectrofluorimetry using a combination of Fura red (34 μM) and fluo-3 (6 μM) as the indicator. Excitation was effected at 490 nm and the emitted light collected at 525 and 660 nm essentially as previously described^{22,23}. Calibration was done by dialysing the cell interior with the indicators and Ca²⁺-EGTA buffers with free Ca²⁺ ranging between 0 and 39.8 μM.

On average (Fig. 2), BAY K8644 (1 μM) increased the integrated Ca²⁺ current by 97 ± 24% (*n* = 4) and potentiated exocytosis by 95 ± 30% (*n* = 6). Subsequent inclusion of forskolin in the continued presence of BAY K8644 elicited a further 188 ± 52% (*n* = 5) potentiation of exocytosis whereas the Ca²⁺ current increased by only +5 ± 7% (*n* = 5). Comparison of the effects of BAY K8644 and forskolin therefore suggests that only 19 ± 5% (*n* = 5) of the effects of cAMP on exocytosis are mediated by an increase in the Ca²⁺ current.

To explore the possibility that cAMP enhances insulin release by a mechanism unrelated to its action on the Ca²⁺ current we next recorded depolarization-evoked Ca²⁺ currents, [Ca²⁺]_i transients and exocytosis before and after intracellular release of cAMP from a caged precursor (Fig. 3*a*). Cyclic AMP had only a small effect on the [Ca²⁺]_i transient but dramatically enhanced secretion: exocytosis increased by 210 ± 60% (*n* = 7) following intracellular application of cAMP whereas the Ca²⁺

transients rose by only 22 ± 9% (Fig. 3*b*).

From the relationship between [Ca²⁺]_i and exocytosis (Fig. 3*c, d*) we estimate that the increase in [Ca²⁺]_i produced by photorelease of cAMP (from 0.49 μM to 0.60 μM; Fig. 3*b*) will produce only a 20% stimulation of exocytosis. This is about 10% of the total increase actually produced by cAMP (210%, see above), implying that changes in [Ca²⁺]_i play only a minor role in mediating the effects of cAMP.

Figure 4*a, b* provides evidence for the existence of a direct effect of cAMP on the exocytotic machinery. Cells voltage-clamped at -70 mV (that is, too negative for Ca²⁺-channel activation) were dialysed with either 0.06 μM (*a*) or 2 μM [Ca²⁺]_i (*b*) and the rate of exocytosis measured before and after photoliberation of 80 μM cAMP. Under basal conditions, the rate of exocytosis averaged 1.5 ± 0.6 fF s⁻¹ (*n* = 5) and 29 ± 6 fF s⁻¹ (*n* = 6; *P* < 0.001) at low and high [Ca²⁺]_i, respectively. After photorelease of cAMP, the corresponding rates

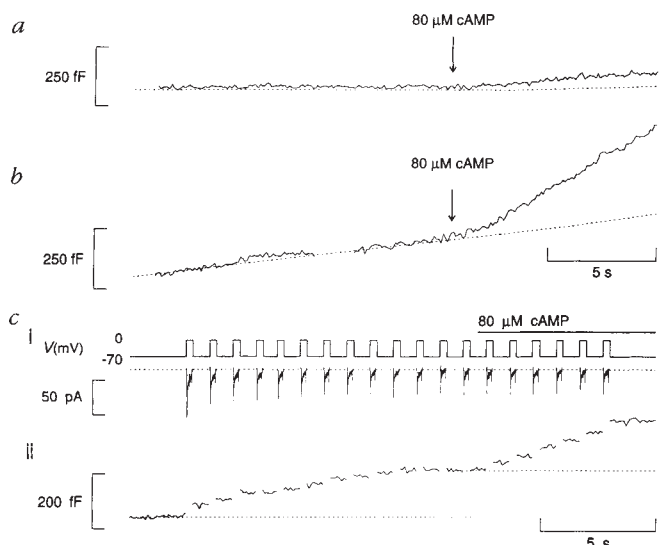


FIG. 4 *a, b*, Stimulation of exocytosis evoked by cAMP in cells dialysed with 0.06 μM (*a*) or 2 μM [Ca²⁺]_i (*b*). In *b*, the recording was interrupted for a few seconds to permit calibration. The cells were held at -70 mV to prevent activation of voltage-gated Ca²⁺ channels. *c*, Simultaneous recordings of membrane potential (*i*, upper), membrane current (*i*, lower) and cell capacitance (*ii*) during a train of 19 depolarizations of 200 ms from -70 to 0 mV. After the 13th pulse, 80 μM cAMP was released from a caged precursor. The dotted line in (*i*) connects the peak amplitude of the Ca²⁺ current elicited by successive depolarizations and indicates the extent of inactivation of the Ca²⁺ current. The horizontal dashed lines in (*ii*) indicate the resting capacitance and the level attained after exhaustion of exocytosis. **METHODS.** In *a* the β-cell was dialysed with an intracellular solution composed of (mM) 125 potassium glutamate, 30 KOH, 3 Mg-ATP, 1 MgCl₂, 2 CaCl₂, 10 EGTA and 5 HEPES-KOH (pH 7.15). The free concentration of Ca²⁺ was calculated to be 0.06 μM. In *b* the cell was dialysed with an intracellular solution consisting of (mM) 125 potassium glutamate, 30 KOH, 3 Mg-ATP, 1 MgCl₂, 9 CaCl₂, 10 EGTA and 5 HEPES-KOH (pH 7.15). The calculated free concentration of Ca²⁺ was 2 μM. The pipette solution in *c* was the same as that in Fig. 3.

of exocytosis increased to $10 \pm 1 \text{ fF s}^{-1}$ (a 6.5-fold increase; $P < 0.001$) and $75 \pm 9 \text{ fF s}^{-1}$ (a 2.6-fold increase; $P < 0.001$) at $0.06 \mu\text{M}$ and $2 \mu\text{M}$ $[\text{Ca}^{2+}]_i$, respectively. Thus cAMP both initiates exocytosis at a Ca^{2+} concentration which by itself does not elicit secretion, and also accelerates exocytosis at higher $[\text{Ca}^{2+}]_i$. These observations are consistent with the hypothesis that cAMP sensitizes the secretory machinery to Ca^{2+} (ref. 16). This may explain the data in Fig. 4c which shows the exocytotic responses during a train of depolarizing voltage pulses. Application of cAMP following exhaustion of exocytosis resulted in a rapid ($< 1 \text{ s}$) reinitiation without any change in the Ca^{2+} current. A possible explanation for this finding is that by increasing the Ca^{2+} sensitivity of exocytosis, cAMP extends the distance from

the Ca^{2+} channels over which secretory granules can be recruited for release.

Our results demonstrate that cAMP, through activation of PKA, potentiates exocytosis from single cells. In the earlier studies^{5,6} it has not been possible to unequivocally distinguish between this possibility and the alternative explanation that cAMP acts by recruitment of additional β -cells. The substrates for PKA remain to be identified¹⁷. Finally, given the similarities between the basic properties of secretion in β -cells and those of neuroendocrine cells and nerve terminals, it might be worth investigating whether a rapid $[\text{Ca}^{2+}]_i$ -independent effect of cAMP also regulates exocytosis in these cells^{18,19}. □

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- Rasmussen, H., Zawlich, K. S., Ganesan, S., Calle, R. & Zawlich, W. S. *Diabetes Care* **13**, 655–666 (1990).
- Pipeleers, D. J., In't Veld, P. A., Maes, E. & van de Winkel, M. *Proc. natn. Acad. Sci. U.S.A.* **79**, 7322–7325 (1982).
- Pipeleers, D. J. *et al.* *Endocrinology* **117**, 824–833 (1985).
- Prentki, M. & Matschinsky, F. M. *Physiol. Rev.* **67**, 1185–1249 (1987).
- Jones, P. M., Salmon, D. M. W. & Howell, S. L. *Biochem. J.* **254**, 397–403 (1988).
- Jones, P. M., Persaud, S. J. & Howell, S. L. *Biochem. J.* **285**, 973–978 (1992).
- Marty, A. & Neher, E. *Proc. natn. Acad. Sci. U.S.A.* **79**, 6712–6716 (1982).
- Joshi, C. & Fernandez, J. *Biophys. J.* **53**, 885–892 (1988).
- Fidler Lim, N., Nowicky, M. C. & Bookman, R. J. *Nature* **344**, 449–451 (1990).
- Horn, R. & Marty, A. *J. gen. Physiol.* **92**, 145–159 (1988).
- Armstrong, D. L. *Trends Neurosci.* **12**, 1–10 (1989).
- Henquin, J. C. *Archiv. Int. Physiol. Biochim.* **93**, 37–48 (1985).
- Ashcroft, F. M. & Rorsman, P. *Prog. Biophys. molec. Biol.* **54**, 97–143 (1989).

- De Wit, R. W. J. *et al.* *Eur. J. Biochem.* **142**, 255–260 (1984).
- Rorsman, P., Ashcroft, F. M. & Trube, G. *Pflügers Arch.* **412**, 587–603 (1988).
- Hughes, S. J., Chalk, J. G. & Ashcroft, S. J. H. *Molec. cell. Endocrinol.* **65**, 35–41 (1989).
- Persaud, S. J., Jones, P. M. & Howell, S. L. *Biochem. biophys. Res. Commun.* **173**, 833–839 (1990).
- Sikdar, S. K., Zorec, R. & Mason, W. T. *FEBS Lett.* **273**, 150–154 (1990).
- Greengard, P., Valtorta, F., Czernik, A. J. & Benfenati, F. *Science* **259**, 780–785 (1993).
- Rorsman, P. & Trube, G. *J. Physiol., Lond.* **374**, 531–550 (1986).
- Ämmälä, C., Bokvist, K., Galt, S. & Rorsman, P. *Biochim. biophys. Acta* **1092**, 347–349 (1991).
- Rorsman, P., Ämmälä, C., Berggren, P.-Ö., Bokvist, K. & Larsson, O. *EMBO J.* **11**, 2877–2884 (1992).
- Ämmälä, C., Eliasson, L., Bokvist, K., Larsson, O., Ashcroft, F. M. & Rorsman, P. *J. Physiol., Lond.* (in the press).

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In utero rearrangements in the trithorax-related oncogene in infant leukaemias

Anthony M. Ford*, Susan A. Ridge*,
Maria E. Cabrera†, Hazem Mahmoud‡,
C. Michael Steel§, Li C. Chan|| & Mel Greaves*

* Leukaemia Research Fund Centre, Institute of Cancer Research, Chester Beatty Laboratories, 237 Fulham Road, London SW3 6JB, UK
† Haematology Section, University of Chile, Santiago, Chile
‡ Department of Hematology/Oncology, St Jude Children's Research Hospital, and the University of Tennessee, Memphis College of Medicine, Memphis, Tennessee 38101-0318, USA
§ MRC Human Genetics Unit, Western General Hospital, Edinburgh EH4 2XU, UK
|| Department of Pathology, Queen Mary Hospital Compound, University of Hong Kong, Hong Kong

THE majority (~75%) of infant acute leukaemias have a reciprocal translocation between chromosome 11q23 and one of several partner chromosomes¹. The gene at 11q23 (named *MLL*, *ALL-1*, *HRX* or *HTRX-1*; refs 2–6) has been cloned and shares homology with the *Drosophila* developmental gene *trithorax*^{3–5}. Rearrangements of this gene (called *HRX* here) occur in introns and cluster in a region of ~10 kb; individual patients have different breakpoints^{3–10}. Here we describe three pairs of infant twins with concordant leukaemia who each share unique (clonal) but non-constitutive *HRX* rearrangements in their leukaemic cells, providing evidence that the leukaemogenic event originates *in utero* and unequivocal support for the intra-placental 'metastasis' hypothesis for leukaemia concordance in twins¹¹.

Three pairs of identical infant twins with acute lymphoblastic leukaemia (ALL) were available for study (Table 1) and we used the P/S4 probe (Fig. 1D) to analyse their leukaemic cell DNA for *HRX* rearrangements. All three pairs of infants had rearrangements of *HRX* (Fig. 1A, B). The crucial observation was that restriction fragments produced with various endonucleases from the DNA of paired twin leukaemia samples co-

TABLE 1 Twin pairs with concordant acute lymphoblastic leukaemia

Twin pair	Diagnosis (subtype)*	Sex†	Age at diagnosis	Karyotype
1a	nALL (L1)	F	9 months	ND
1b	nALL (L1)	F	10 months	ND
2a‡	ALL§	M	5 weeks	46, XY
2b	ALL	M	5 weeks	46, XY
3a	nALL (L1)	F	2 months	46, XX, t(11;19)(q23;p13)
3b	nALL (L1)	F	2 months	46, XX, t(11;19)(q23;p13)

M, male; F, female; ND, not determined.

* Null (n) or progenitor B ALL: DR⁺, CD10⁺, CD19⁺, T/myeloid markers negative (except for pair 3, who were myeloid marker CD33⁺). L1 represents small lymphoblast morphological subtype FAB (French-American-British) classification³³. Detailed clinical and haematological case reports of pairs 1 and 3 are being prepared for publication elsewhere.

† Criteria for monozygosity in these cases were identity of HLA and blood group, plus a single monochorionic placenta at birth.

‡ Patients diagnosed in 1976. Samples stored since then as blood samples in liquid nitrogen (case 2a) and testicular biopsy with leukaemic infiltration (case 2b).

§ Immunophenotyped as DR⁺, E/CD2⁺ in 1976 (by M.G.). Likely to be progenitor B/null, ALL.

|| Karyotyped by non-banding methods in 1976. Note that this apparently normal karyotype does not exclude the presence of a translocation or rearrangement involving 11q23 (refs 1, 10).

migrated but were different between twin pairs and were also different from the controls (Fig. 1A, B). The rearrangements were also distinct from eight other infant 11q23 translocations analysed in our laboratory⁷. Other recent descriptions of the *HRX* rearrangements also reveal a diversity of breakpoints^{2–10}. These data provide strong evidence that the rearrangements in paired twins are identical.

Evidence that the rearrangements detected were not inherited as structural abnormalities or restriction site polymorphisms is provided by the observation that cells transformed by Epstein-Barr virus (EBV) and taken from patient 2a, remission blood mononuclear cells from pair 1a and b and from 3b, liver (post mortem) from twin 3a (Fig. 1C) and maternal and paternal blood DNA from pairs 1 and 3 (data not shown), all lacked the

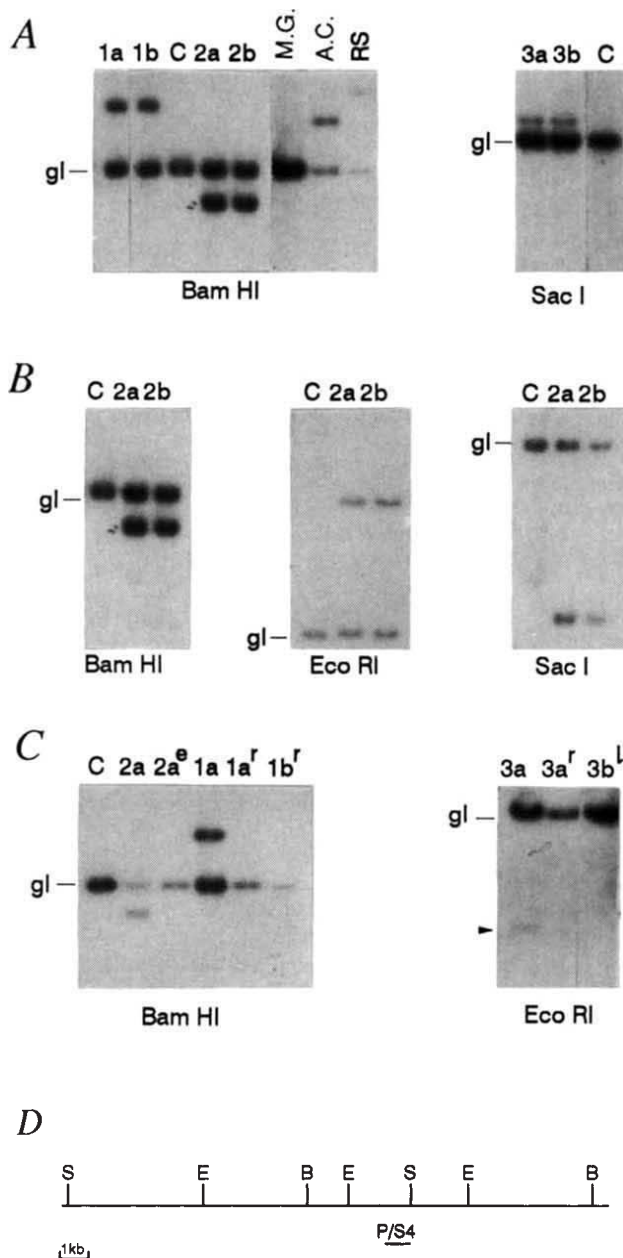


FIG. 1 Analysis of breakpoint specificity at chromosome 11q23 in twin leukaemias and controls. 2 μ g of DNA isolated from twin pair leukaemic cells from bone marrow, blood or, in the case of twin 2b, testicular biopsy, digested with the restriction enzymes indicated and hybridized to the P/S4 probe^{7,10} as described^{7,34}. **A**, Individual twin pairs are referred to by number. A.C. (patient) and RS (cell line³⁵) are positive controls with known 11q23 rearrangements, C is a germ-line (gl) negative control (KG1 cells). M.G. is a patient sample (ALL) with no 11q23 rearrangement (used as an additional negative control). **B**, DNA from one set of twins (case 2a,b) digested with three different enzymes to confirm the identical breakpoint. Co-migration of restriction fragments generated by two enzymes was also demonstrated for twin pair 3 (data not shown). Insufficient DNA was available from both twin 1a and 1b to confirm identity with multiple enzymes. **C**, The break at 11q23 is acquired and not inherited. DNA from each set of twins was digested with *Bam*HI or *Eco*RI and hybridized as described. The superscript e indicates that DNA is from EBV-transformed cells from patient 2a, r that DNA is from remission blood from patients 1a, 1b and 3a, and l that DNA is from post-mortem liver of patient 3b. Each sample lacks the rearrangement seen in the corresponding leukaemic cells. **D**, Restriction map of the P/S4 probe is shown.

rearrangement observed in the corresponding leukaemic cell DNA. We therefore conclude that the rearrangements of *HRX* were acquired and not inherited. As the rearrangement that is shared by each twin pair is both unique and acquired, the explanation must be that it arose as a single clonal event *in utero* in one twin, generating leukaemic or pre-leukaemic clonogenic progeny, which metastasized to the other twin through vascular anastomoses in their shared monochorionic placenta.

We sought further evidence for this interpretation by analysis of two other markers of clonal origin that might be expected to be informative. Rearrangements of immunoglobulin and T-cell receptor genes provide unique clonal markers in lymphoid malignancies¹². Three patterns of IgH gene rearrangements were seen in the infant twins (Fig. 2). In one pair (pair 3), an identical oligoclonal pattern of rearrangements was present. In pair 1, rearrangements were biallelic, with one shared and one different rearranged allele. In both pairs 3 and 1, common allelic rearrangements were confirmed using other restriction enzymes. These data confirm the common clonal and *in utero* origins of the leukaemias in twin pairs 1 and 3. Pair 2, in contrast to the others, had biallelic but distinct rearrangements. This result, while not confirming a common clonal origin for the leukaemic cell populations in twin pair 2, does not provide contrary evidence because IgH rearrangements may continue to diversify within a single clone of transformed leukaemic B-cell progenitors^{13,14}.

Clonality of cancer cells can also be analysed in females that are heterozygous for X-linked polymorphisms¹⁵. Two of the three pairs of twins were female and both pairs were found to be heterozygous at the phosphoglycerate kinase (PGK) locus. All four individual leukaemic cell populations, as expected, expressed a single allele, indicating monoclonality (Fig. 3). More significantly, the individual twin samples in each pair expressed the same (1.7-kilobase) parental allele. This result is consistent with the paired samples being derived from the same single clone (Fig. 3 legend).

Concordance of leukaemia in twins cannot be explained in these cases, or those previously reported^{16,17}, by known risk factors for leukaemia, such as familial leukaemia or cancer (that is, an inherited predisposition), diagnostic X-rays during pregnancy, consanguinity or maternal age¹⁸. The suggested¹¹

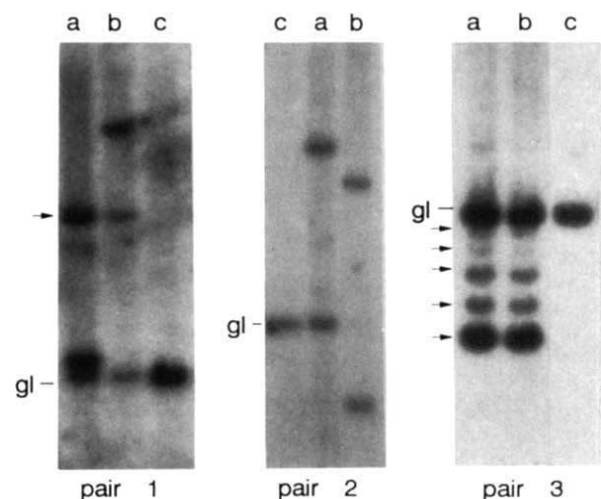


FIG. 2 Comparison of IgH gene rearrangements between identical twins. DNA (2 μ g) isolated from twin pairs was digested with *Bgl*II and hybridized to a JH region probe as described³⁴. The size of the germ-line (gl) fragment (lane c) for *Bgl*II digests is 3.3 kb. Arrows indicate shared bands. Pair 1: biallelic monoclonal pattern with one shared band; pair 2: biallelic monoclonal pattern with no shared bands; pair 3: oligoclonal pattern with all bands shared. Note that one other case of twin infant acute lymphoblastic leukaemias with a shared allelic IgH rearrangement has been reported³⁶.

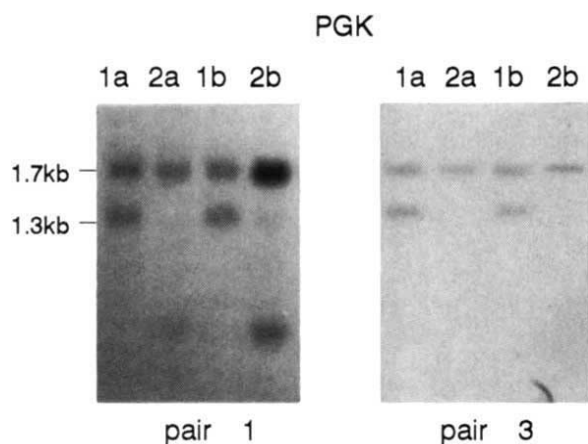


FIG. 3 Clonality and X chromosome inactivation. Leukaemic cell population clonality was determined by pattern of X-chromosome inactivation using 5' PGK probe pSPT19 as described^{15,37}. The principle involved in using the PGK polymorphism as an assay for clonality is that the expressed parental allele is methylated and, as a consequence, will be protected from digestion by methylation-sensitive restriction enzymes that cut at CpG sites³⁷. If one of the parental alleles is preferentially protected according to this criterion, then the cell population is highly likely to be monoclonal. DNA (4 µg) from each twin (pairs 1 and 3) was digested simultaneously with *EcoRI*, *BglI* and *BglII* and then divided in half. One aliquot was not digested any further (lanes 1a and 1b), the other aliquot was further digested with the methylation-sensitive enzyme *HpaII* (lanes 2a and 2b). Southern blot hybridization (with the PGK probe) was carried out as described³⁴. In each of the four leukaemic samples, a single X allele is active/inactive, a result that is compatible with monoclonality of the cell population. For each twin pair, the same (1.7 kb) allele is active, a result that is consistent with the paired leukaemias arising from a single clone *in utero*. Note, however, that concordance of parental allele usage could have arisen by chance, given that there is a 50:50 possibility of either allele being expressed in a clonal population. Lack of concordance of alleles would in this respect have been more informative and the value of this experiment therefore lies principally in the fact that it fails to contradict the common clonal origin of *HRX* rearrangements in twin infant leukaemias.

and now proven explanation that these leukaemias arise from a single cell clone *in utero* in one twin and subsequently spread to the other twin by their shared placental circulation accommodates the observations that a high rate of concordance of leukaemia in twins is restricted to monozygotic pairs and is only very rarely observed in other paediatric cancers^{16,19,20} which are not primarily blood borne. The metastasis hypothesis for twin leukaemia is also supported by cytogenetic studies showing similar chromosome abnormalities in the leukaemic cells from each twin²¹⁻²³. This earlier evidence is weakened, however, by incomplete description of karyotypes and the more recent appreciation that similar types of acute leukaemia in unrelated individuals may share acquired chromosome abnormalities that appear identical at the level of karyotype, with 11q23⁻ in infant leukaemia being a prime example¹.

An accurate concordance rate of leukaemia in identical twins is unknown but is estimated to be at least 25% for infants (<12 months) and declines with age¹⁶. As only 50-60% of identical twins have a monochorionic placenta²⁴ and therefore the anatomical potential for leukaemic spread, concordance rate for monozygous monochorionic infant twins is probably between 50% and 100%. This estimate is relevant to the question of whether it is reasonable to extrapolate from the unusual situation of twins with leukaemia described here to non-twin infants with leukaemia. It could be argued that by selecting concordant pairs of twins for study, there is a selective bias in favour of leukaemias initiated *in utero* and that these might be atypical. This potential criticism loses force when it is appreciated that twins are no more at risk of leukaemia than non-twin

individuals and that the concordance rate in twins is very high for infant ALL. We therefore draw the conclusion that in the majority of cases of non-twin infant acute leukaemia, the commonly observed chromosome translocations that disrupt *HRX* (refs 1, 25) also occur during pregnancy. The very high concordance rate, the relatively short latency that follows *HRX* rearrangement *in utero*, and the synchrony of post-natal clinical onset in identical twins (our data, and ref. 16), also suggest that rearrangement of *HRX* is either sufficient for leukaemia development or renders pre-leukaemic cells at very high risk of acquiring the necessary secondary mutations. These interpretations accord with the observation that acute leukaemias with 11q23 rearrangements not only predominate in infants under one year of age but are sometimes clinically diagnosed as congenital leukaemias at birth^{26,27}. 11q23 translocations do occur sporadically in older children and adults²⁸; the developmental timing of *HRX* rearrangements in these older patients has not been ascertained and need not be the same as in infants.

These findings have aetiological and epidemiological implications. Although the data do not formally exclude an inherited component to leukaemogenesis in infants, they focus attention on possible causative events during pregnancy. In this respect, it is interesting that chromosome translocations involving 11q23 are very common in secondary acute myeloid leukaemias arising in cancer patients previously treated with epidophylotoxins such as etoposide/VP-16 (refs 29-31), which induce and/or stabilize DNA breaks by a topoisomerase II-dependent mechanism³². Similar molecular mishaps could arise during pregnancies that precede infant acute leukaemia. □

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- Pui, C.-H., Crist, W. M. & Look, A. T. *Blood* **76**, 1449-1463 (1990).
- Zieman-Van Der Poel, S. et al. *Proc. natn. Acad. Sci. U.S.A.* **88**, 10735-10739 (1991).
- Gu, Y. et al. *Cell* **71**, 701-708 (1992).
- Tkachuk, D. C., Kohler, S. & Cleary, M. L. *Cell* **71**, 691-700 (1992).
- Djabali, M. et al. *Nature Genet.* **2**, 112-118 (1992).
- Cimino, G. et al. *Cancer Res.* **51**, 6712-6714 (1991).
- Morgan, G. J. et al. *Blood* **80**, 2172-2175 (1992).
- McCabe, N. R. et al. *Proc. natn. Acad. Sci. U.S.A.* **89**, 11794-11798 (1992).
- Yamamoto, K. et al. *Oncogene* **8**, 479-485 (1993).
- Chen, C.-S. et al. *Blood* (in the press).
- Clarkson, B. D. & Boyse, E. A. *Lancet* (ii), 699-701 (1971).
- Korsmeyer, S. J. et al. *J. clin. Invest.* **71**, 301-313 (1983).
- Carter, M., Neale, G. A. M. & Kitchingman, G. R. *Leukemia* **5**, 668-672 (1991).
- Bird, J., Galili, N., Link, M., Stites, D. & Sklar, J. *J. exp. Med.* **168**, 229-245 (1988).
- Fearon, E. R. et al. *New Engl. J. Med.* **315**, 15-24 (1986).
- Zuelzer, W. W. & Cox, D. E. *Sem. Hematol.* **22B**, 228-249 (1969).
- Miller, R. W. *J. natn. Cancer Inst.* **46**, 203 (1971).
- Lin, M. S. *The Leukemias: Epidemiologic Aspects* Vol. 6 (Oxford University Press, New York, 1985).
- Draper, G. J., Heaf, M. M. & Kinnier Wilson, L. M. *J. med. Genet.* **14**, 81-90 (1977).
- Miller, R. W. *New Engl. J. Med.* **279**, 122-126 (1968).
- Hilton, H. B., Lewis, I. C. & Trowell, H. R. *Blood* **35**, 222-226 (1970).
- Chaganti, R. S. K., Miller, D. R., Meyers, P. A. & German, J. *New Engl. J. Med.* **300**, 1032-1034 (1979).

- Hartley, S. E. & Sainsbury, C. *Hum. Genet.* **58**, 408-410 (1981).
- Strong, S. J. & Corney, G. *The Placenta in Twin Pregnancy* (Pergamon, Oxford, 1967).
- McCabe, N. R. et al. *Proc. natn. Acad. Sci. U.S.A.* **89**, 11794-11798 (1992).
- Van Den Berghe, H. et al. *Hum. Genet.* **46**, 173-180 (1979).
- Prigogina, E. L. et al. *Hum. Genet.* **53**, 5-16 (1979).
- Pui, C.-H. *Leukemia Lymphoma* **7**, 173-179 (1992).
- Ratain, M. J. et al. *Blood* **70**, 1412-1417 (1987).
- Pui, C.-H. et al. *New Engl. J. Med.* **321**, 136-142 (1989).
- Rubin, C. M. et al. *Blood* **78**, 2982-2988 (1991).
- Ross, W. et al. *Cancer Res.* **44**, 5857-5860 (1984).
- Bennett, J. M. et al. *Brit. J. Haemat.* **33**, 451-458 (1976).
- Ford, A. M., Molgaard, H. V., Greaves, M. F. & Gould, H. J. *EMBO J.* **2**, 997-1001 (1983).
- Stong, R. C. et al. *Blood* **65**, 21-31 (1985).
- Pombo de Oliveira, M. S. et al. *Lancet* **ii**, 969-970 (1986).
- Fearon, E. R. et al. *New Engl. J. Med.* **316**, 427-431 (1987).

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Leukaemia inhibitory factor is necessary for maintenance of haematopoietic stem cells and thymocyte stimulation

Jean-Louis Escary*, Jacqueline Perreau*, Dominique Duménil†, Sophie Ezine‡ & Philippe Brûlet*§

* Unité d'Embryologie Moléculaire de l'Institut Pasteur, URA 1148 du CNRS, 75724 Paris Cedex 15, France

† U362 de l'INSERM, Institut Gustave Roussy, 94800 Villejuif, France

‡ U345 de l'INSERM, CHU Necker, 75015 Paris Cedex 15, France

§ To whom correspondence should be addressed.

LEUKAEMIA inhibitory factor (LIF) has a variety of effects on different cell types *in vitro*¹, inhibiting the differentiation of embryonic stem cells^{2,3} and promoting the survival and/or proliferation of primitive haematopoietic precursors^{4,5} and primordial germ cells^{6,7}. Here we show that LIF-deficient mice derived by gene targeting techniques have dramatically decreased numbers of stem cells in spleen and bone marrow. Injection of spleen and marrow cells from these mice promotes long-term survival of lethally irradiated wild-type animals, however, showing that the LIF⁻ stem cells remain pluripotent. The numbers of committed progenitors are also reduced in the spleen but not the bone marrow, suggesting that stem cells interact differently with the splenic and medullary

microenvironment. Heterozygous animals are intermediate in phenotype, implying that LIF has a dosage effect, and defects in stem cell number can be compensated by exogenous LIF. LIF thus appears to be required for the survival of the normal pool of stem cells, but not their terminal differentiation.

LIF⁻ mice were generated by deleting a 3.3-kilobase (kb) DNA fragment containing all the known exons and introns plus a part of the 3' untranslated region of the gene (Fig. 1). This DNA fragment codes for the two forms of the murine LIF

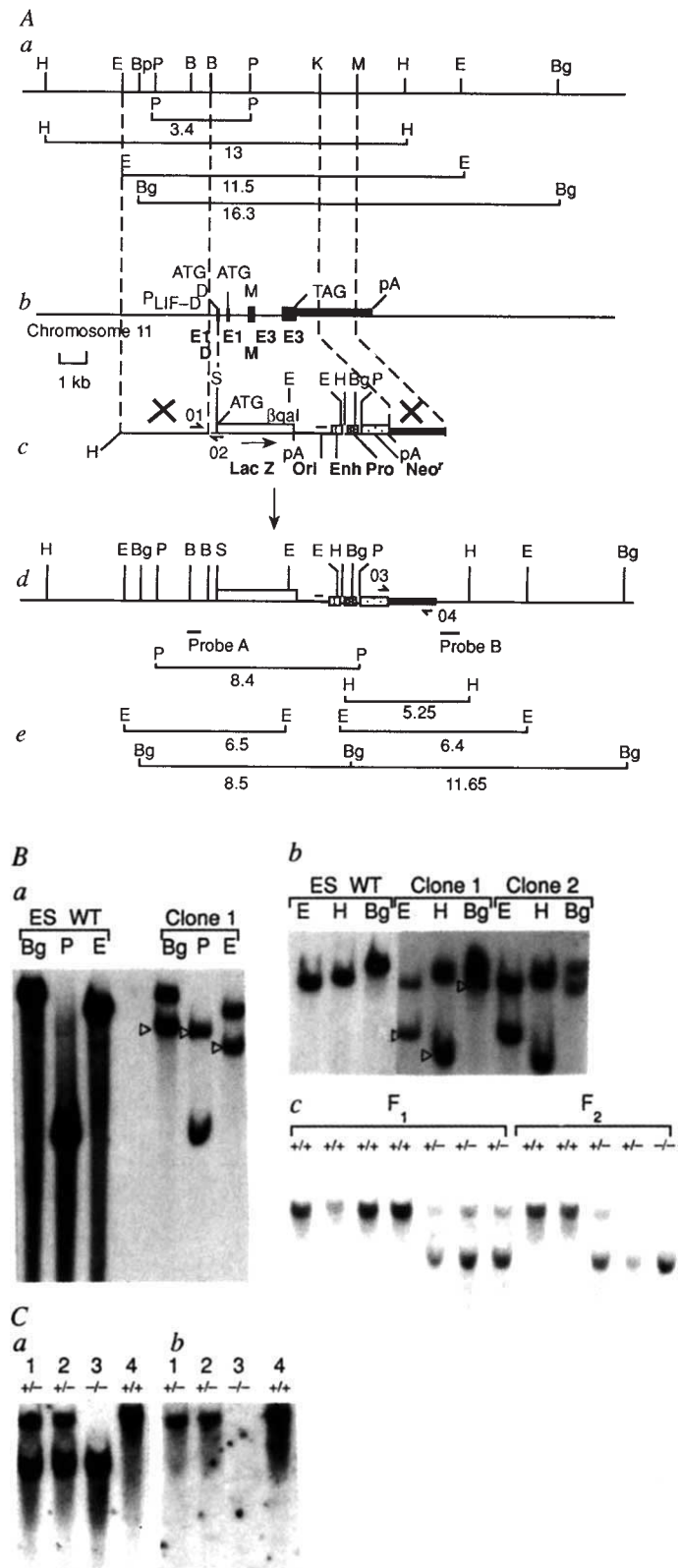
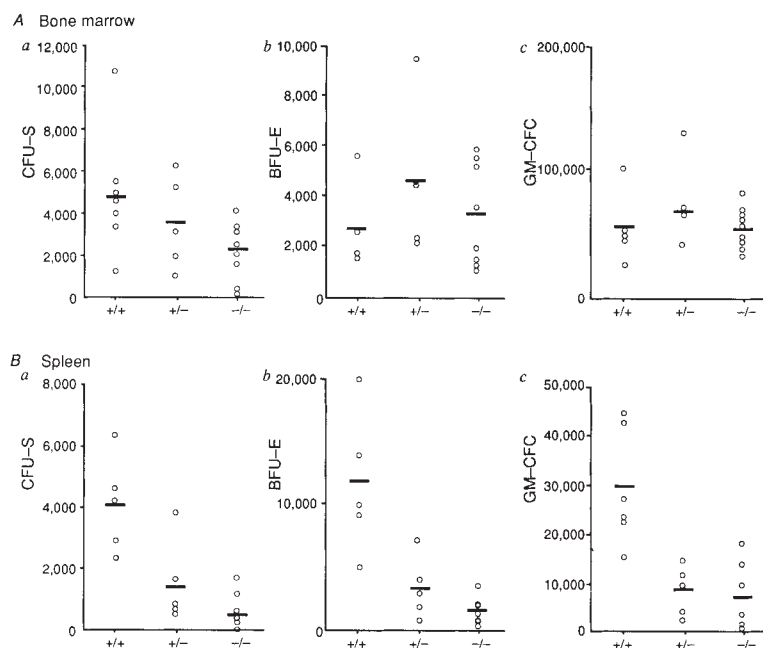


FIG. 1 **A**, Targeting strategy for substituting the murine LIF coding region with the reporter gene *LacZ* in (129/Sv) ES cells^{18,19}. A 3,301-bp DNA fragment of the gene including all the known introns and exons of the two forms of LIF (soluble LIF-D and matrix-associated LIF-M) plus 571 bases of the common 3' untranslated region was deleted. Mutated ES cell clones were characterized by Southern blotting with 5' (probe A) and 3' (probe B) genomic LIF probes (see **B**, **a** and **b**). After targeting, the 5'-most ATG was provided by *LacZ* and the junction with the LIF diffusible promoter was verified by sequencing. Mutated ES clones were microinjected into C57Bl/6 blastocysts, which were implanted into pseudopregnant females. Clone 1 gave 6 chimaeric animals, from which two males yielded germ-line transmission of the mutation by interbreeding with F₁ (C57Bl/6 × DBA/2) females. **a**, Partial restriction map of the murine LIF locus. *Bam*HI (B), *Pst*I (P), *Hind*III (H), *Eco*RI (E), *Bgl*II (Bg), *Kpn*I (K), *Mbo*I (M). **b**, Exon positions on the mouse chromosome 11 (ref. 20) (black bars) relative to the map in **a**. P_{LIF-D} denotes the diffusible LIF promoter. Positions are shown of the first exons (E1_D, E1_M) and initiation codons (ATG_D, ATG_M) of the two forms of LIF, the termination codon (TAG), a 3'-untranslated region (3.2 kb) and the polyadenylation site (pA)²¹. **c**, Replacement vector (linearized at *Nsi*I site, N) containing the cloning vector pGN¹⁸ and two flanking regions of the LIF coding region. The 5' LIF fragment (3,853 bp; generated in part by polymerase chain reaction (PCR) using primers 01 and 02), together with the 3' LIF 1,422-bp fragment were subcloned into pGN¹⁸. **d**, Predicted structure of the targeted LIF locus. Half-arrows indicate primers 03 (5'-AACTTCCTCTCTGCTATTC-3') and 04 (5'-AGGTAAAGTGCTTAGAGAGTG-3') used for PCR detection of targeted events in ES cells (data not shown). **e**, Predicted sizes (kb) of targeted DNA fragments detected by probes A and B. The latter does not recognize the replacement vector but probe A does. **B**, Southern analysis of the LIF locus in targeted ES (129/Sv) cells and mutant animals. Recombinant DNA fragments are indicated by triangles. **a**, Southern analysis of *Bgl*II-, *Pst*I-, and *Eco*RI-digested genomic DNA from wild-type and targeted (clone 1) ES cells hybridized to probe A. **b**, Southern analysis of *Eco*RI-, *Hind*III-, *Bgl*II-digested genomic DNA from wild-type ES cells and the LIF-targeted ES clones 1 and 2 using probe B. **c**, Southern analysis of *Eco*RI-digested genomic DNA from an F₁ litter obtained by interbreeding a male chimaera (derived with clone 1) and a female (C57Bl/6 × DBA/2) and from an F₂ litter obtained by crossing heterozygous animals. The membrane was hybridized with probe B. **C**, Confirmation of the LIF coding region deletion by Southern analysis of *Eco*RI-digested genomic DNA from four F₂ mice. The membrane was hybridized to **a**, probe B, then stripped and rehybridized to **b**, an entire cDNA_{LIF-D} probe²².

FIG. 2 Numbers of haematopoietic progenitors (a, CFU-S; b, BFU-E; c, GM-CFC) in A, the bone marrow, and B, the spleen of 5 to 9 mice of each genotype (wild type, +/+; heterozygote, +/-; homozygote, -/-).

METHODS. Bone marrow cells were removed from femurs and tibias. Bone marrow and spleen cells were dissociated and suspended in Iscove's modified Dulbecco's medium (IMDM). CFU-S numbers were determined as described in ref. 23. 10^5 bone marrow and 10^6 spleen cells were injected into lethally irradiated mice and macroscopic spleen colonies were counted 12 days after injection. The number of committed progenitor cells (BFU-E and GM-CFC) was determined by a methyl cellulose culture technique²⁴. Briefly, 10^5 or 10^6 bone marrow and spleen cells were used to determine BFU-E, and 5×10^4 and 5×10^5 bone marrow and spleen cells were used to determine GM-CFC. The culture mixture was composed of (0.8%) methyl cellulose, 20% fetal calf serum and 20% growth factors (Wehi-3B conditioned medium plus erythropoietin 1 U ml^{-1} for BFU-E, and lung and heart conditioned medium for GM-CFC). Culture mixtures were plated in quadruplicate in 35-mm-diameter Petri dishes and incubated at 37°C in a humidified atmosphere with 5% CO_2 for 10 days and 7 days for BFU-E and GM-CFC, respectively. For CFU-S in the bone marrow of homozygotes, $P \leq 0.01$; for CFU-S, BFU-E and GM-CFC in the spleen of homozygotes, $P \leq 0.002$; for CFU-S, BFU-E and GM-CFC in the spleen of heterozygotes, $P \leq 0.05$ (t-test).



protein⁸, the soluble form and the matrix-associated form. LIF⁻ animals appeared to be normal at birth and afterwards. Histological analysis of most organs did not disclose any abnormalities. In particular, the thymuses and spleens of homozygotes were normal in size. LIF⁻ mice were somewhat smaller than wild-type mice and, to a lesser extent, this was also true of heterozygous animals. Males were fertile but homozygous females were sterile, in agreement with a report⁹ in which transplantation of LIF⁻ blastocysts into wild-type pseudopregnant females indicated that maternal LIF expression is necessary for blastocyst implantation.

By examining progenitor cells in the spleen and the bone marrow, we found a dramatic decrease in several stem cell compartments in the spleen: pluripotent stem cells (colony-forming unit (CFU)-S) were reduced by 88%, committed eryth-

roid (burst-forming unit (BFU)-E) progenitors by 85%, and granulocyte-macrophage (GM-colony-forming capacity (CFC)) progenitors by 73% (Fig. 2). The numbers of progenitors in the spleen of heterozygous mice were intermediate between those of wild-type and homozygous mutant animals, indicating a dosage effect for the LIF gene product. In the bone marrow of homozygotes, the CFU-S number was significantly decreased, but only by 59%. GM-CFC and BFU-E compartments in bone marrow were less affected. The total nucleated cell numbers in the spleen ($177.0 \pm 10 \times 10^6$) and the bone marrow ($27.6 \pm 1.9 \times 10^6$ per leg) of LIF⁻ mice were normal. A CFU-S activity is associated with a subset of haematopoietic progenitors defined by the markers Sca⁺Thy1⁺Lin⁻ (ref. 10). We found that their numbers in the spleens and bone marrow of LIF⁻ mice were normal. These data suggest that the CFU-S pool is not

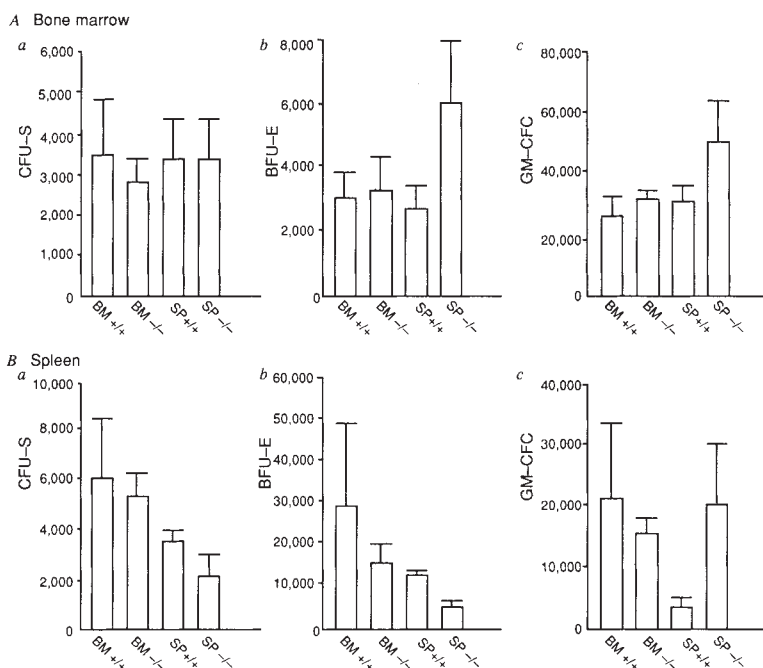


FIG. 3 Number of haematopoietic progenitors (CFU-S, BFU-E and GM-CFC) per leg (A) or spleen (B) of 3 mice grafted with cells from bone marrow (BM) or spleen (SP) of homozygous LIF mutant (-/-) or wild-type mice (+/+).

METHODS. The number of nucleated cells injected was adjusted so that grafts contained roughly the same number of CFU-S (arbitrarily, 16.0 ± 1.0). Fifteen recipient mice were used per group. 38 days after the graft, 3 mice from each group were killed and the haematopoietic progenitor content (CFU-S, BFU-E and GM-CFC) of their bone marrow and spleen determined as described in Fig. 2 legend. The proportion of donor-type was over 75% in the 12 animals grafted in sex-mismatched condition as assessed on splenic and medullary DNAs with a Y-chromosome-specific probe (not shown).

homogeneous and that the LIF deficiency may affect specific cells in it. A different effect of LIF on haematopoiesis in spleen and bone marrow has been noted in gain-of-function experiments¹¹. The spleen was enlarged with increased numbers of progenitor cells, whereas bone marrow populations were less affected. In contrast to the decrease in the number of bone marrow CFU-S and spleen CFU-S, BFU-E and GM-CFC, in LIF⁻ mice the levels of circulating mature red and white blood cells and of blood platelets^{11,12} ($1,040 \pm 145 \times 10^6$ per ml) were normal. Therefore LIF maintains the pool of haematopoietic stem cells *in vivo* and does not promote their terminal differentiation, which requires only a small number of stem cells for steady-state haematopoiesis.

To determine whether LIF⁻ stem cells could differentiate normally, as indicated by the normal level of circulating mature cells in LIF⁻ mice, we tested the ability of mutant primitive haematopoietic stem cells to reconstitute a functional haematopoietic system. Bone marrow and spleen cells from LIF⁻ and wild-type male mice were injected into lethally irradiated wild-type female mice. The same number of CFU-S (16.0 ± 1.0) were injected in each case. Wild-type marrow and spleen cells promoted survival (monitored for four months) of 80 and 93% of the recipients, respectively, and LIF⁻ bone marrow and spleen cells promoted survival of 86 and 70% of the animals, respectively. Thirty-eight days after the graft, the committed progenitor content in the bone marrow and the spleen of some of the grafted animals was analysed (Fig. 3). When bone marrow or spleen cells from LIF⁻ mice were used, the numbers of CFU-S, BFU-E and GM-CFC in bone marrow were equivalent to those from wild-type cells, whereas spleens contained slightly fewer CFU-S and BFU-E. The high proportion of mice surviving 4 months after receiving either LIF⁻ bone marrow or spleen cells argues against any gross defect in the ability of LIF⁻ stem cells to reconstitute a functional haematopoietic system. So LIF appears to be required in the microenvironment to maintain stem cell numbers, rather than affecting the potential of the haematopoietic stem cells. The spleen may function as a storage site for early progenitors whose survival is under the control of LIF.

We investigated whether exogenous LIF could compensate for the mutation by implanting LIF⁻ mice ($n=3$) with miniosmotic pumps (ALZET, model 2001) delivering recombinant (r) LIF at a rate of 10^3 IU per hour. After 7 days of LIF treatment, two mice had normal numbers of bone marrow (88 and 120% of the control CFU-S value) and spleen progenitors (90 and 104% of CFU-S, 50 and 70% of BFU-E, 130 and 150% of GM-CFC control values), and one had partially recovered: only splenic CFU-S (53% of the control value) and GM-CFC (60% of the control value) were increased. Treatment with a higher dose of rLIF (10^4 IU) caused haematopoietic disorders in LIF⁻ mice, such as strong thymic atrophy and a reduction in Sca⁺Thy1⁺Lin⁻ subset.

Lymphoid cells were also examined in LIF⁻ mice. B-cell subsets in the bone marrow, spleen and peritoneum were normal, as were IgM, IgG and IgA secretions (not shown). In the thymus, the average total cell number was similar in normal and in LIF⁻ mice (67.7×10^6 ($n=11$) versus 49.7×10^6 ($n=12$)). Furthermore, no major differences in thymocyte subsets were detected using monoclonal antibodies raised against CD4, CD8 and CD3 (not shown). Nevertheless, thymic T-cell response to concanavalin A or allogeneic stimulation was dramatically reduced in heterozygous and homozygous (6–8 weeks old) mice (Fig. 4a), but peripheral T-cell responses were normal (not shown). As LIF is expressed by thymic epithelial cells¹³, the impairment of thymic T-cell stimulation in LIF mutant mice may reflect defects in their microenvironment, for example in epithelial cells and/or macrophages. Accordingly, in the reconstitution experiments thymic T-cell responses from recipient animals (wild-type environment) were normal, irrespective of whether the genotype of the grafted marrow or spleen cells was wild-type or LIF⁻

(Fig. 4b).

In LIF⁻ animals, the CFU-S pool is diminished but can be restored by exogenous LIF. The pool could be homogeneously diminished or only specific subpopulations might be affected. LIF⁻ primitive haematopoietic stem cells can reconstitute the haematopoietic system in wild-type recipient animals, which suggests that these cells, which are different from day-12 CFU-S, are functional in this environment, although we cannot exclude the possibility that their numbers are also decreased in LIF⁻ mice. Our results indicate that the LIF null mutation primarily affects the LIF-producing microenvironment rather than the haematopoietic stem cell potential. It is unclear whether LIF acts directly or stimulates another stromal cell population to produce different factors involved in the maintenance of haematopoietic stem cells. During embryogenesis the first stem cells of the haematopoietic system appear in the blood islands in the extra-embryonic part of the embryo. We have shown that

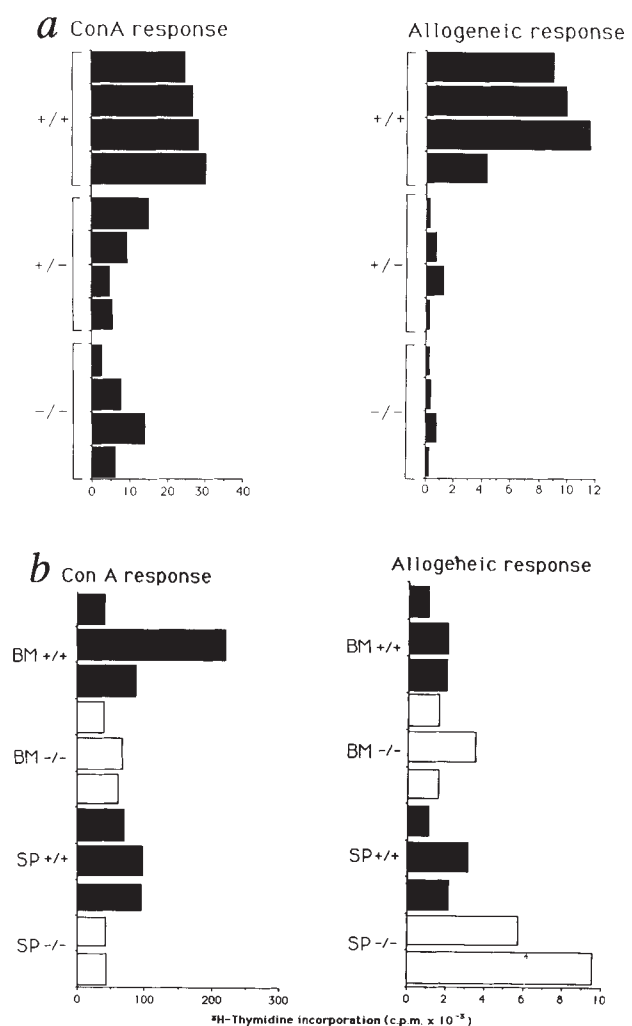


FIG. 4 **a**, Inability of thymic T cells from LIF-deficient mice to generate a proliferative response after concanavalin A (ConA) and allogeneic stimulation. Four animals of each genotype (+/+, +/-, -/-) are represented and data-averaged from three separate experiments. Values represent the mean of triplicate cultures. **b**, Lethally irradiated mice reconstituted with wild-type (+/+) or mutant (-/-) bone marrow (BM) or spleen (SP) cells; 38 days later, thymic T-cell responses to ConA and allogeneic stimulations were determined. 2 or 3 animals from each group are represented. METHODS. Thymocytes (4×10^5 per well) were cultured in 0.2 ml IMDM/10% FCS in 96-well flat-bottomed trays together with ConA ($5 \mu\text{g ml}^{-1}$) or 10^6 irradiated ($3,000$ rads) splenocytes from C₃H/HeJ^(H-2k) mice. They were pulsed with $1 \mu\text{Ci } ^3\text{H-thymidine}$ on day 2 for ConA stimulation, or on day 3 for allogeneic stimulation, and collected 4 h later.

LIF is transcribed in these extra-embryonic tissues¹⁴, providing a local source of LIF for the stem cells. Be they adult or embryonic, stem cells can self-renew or differentiate depending on the stimuli. According to our *in vivo* results, LIF would support survival and/or self-renewal of haematopoietic stem cells *in vivo*. In an LIF-deficient environment, those stem cells will tend to differentiate or disappear. Concerning the other suspected stem cell target of LIF activity (embryonic, primordial germ)^{2,3,6,7}, LIF deficiency *in vivo* could be partially compensated by one or more of the members of the LIF cytokine family, oncostatin-M, interleukin-6, granulocyte colony-stimulating factor and CNTF^{15,16}. In addition, supporting our interpretation of the function of LIF *in vivo*, we have demonstrated that LIF overexpression *in vivo* inhibits differentiation of primitive ectodermal cells¹⁷ into mesoderm. □

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1. Hilton, D. J. & Gough, N. M. *J. Cell Biochem.* **46**, 21–26 (1991).
2. Smith, A. G. *et al. Nature* **336**, 688–690 (1988).
3. Williams, R. L. *et al. Nature* **336**, 684–687 (1988).
4. Fletcher, F. A. *et al. J. exp. Med.* **174**, 837–845 (1991).

5. Leary, A. G., Wong, G. G., Clark, S. C., Smith, A. G. & Ogawa, M. *Blood* **75**, 1960–1964 (1990).
6. De Felici, M. & Dolci, S. *Dev. Biol.* **147**, 281–284 (1991).
7. Matsui, Y. *et al. Nature* **353**, 750–752 (1991).
8. Ratjen, P. D., Toth, S., Willis, A., Heath, J. K. & Smith, A. G. *Cell* **62**, 1105–1114 (1990).
9. Stewart, C. L. *et al. Nature* **359**, 76–79 (1992).
10. Spangrude, G. J. *et al. Blood* **78**, 1395–1402 (1991).
11. Metcalf, D., Nicola, N. & Gearing, D. P. *Blood* **76**, 50–56 (1990).
12. Metcalf, D., Hilton, D. & Nicola, N. A. *Blood* **77**, 2150–2153 (1991).
13. Le, P. T. *et al. J. Immunol.* **145**, 3310–3315 (1990).
14. Conquet, F. & Brûlet, P. *Molec. cell. Biol.* **7**, 3801–3805 (1990).
15. Rose, T. M. & Bruce, A. G. *Proc. natn. Acad. Sci. U.S.A.* **88**, 8641–8645 (1991).
16. Patterson, P. H. *Curr. Neurobiol.* **2**, 94–97 (1992).
17. Conquet, F., Peyrieras, N., Tîret, L. & Brûlet, P. *Proc. natn. Acad. Sci. U.S.A.* **89**, 8195–8199 (1992).
18. Le Mouellîc, H., Lallemand, Y. & Brûlet, P. *Proc. natn. Acad. Sci. U.S.A.* **87**, 4712–4716 (1990).
19. Le Mouellîc, H., Lallemand, Y. & Brûlet, P. *Cell* **69**, 251–264 (1992).
20. Kola, I., Davey, A. & Gough, N. M. *Growth Factors* **2**, 235–240 (1990).
21. Stahl, J. *et al. J. Biol. Chem.* **265**, 8833–8841 (1990).
22. Gearing, D. P., King, J. A. & Gough, N. M. *Nucleic Acids Res.* **16**, 9857 (1988).
23. Till, J. E. & McCulloch, E. A. *Radiat. Res.* **14**, 213–222 (1961).
24. Worton, R. G., McCulloch, E. A. & Till, J. E. *J. cell. Physiol.* **74**, 171–182 (1969).

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A non-receptor tyrosine kinase that inhibits the GTPase activity of p21^{cdc42}

Edward Manser*, Thomas Leung*, Harfizah Salihuddin*, Lydia Tan* & Louis Lim*†

* Institute of Molecular and Cell Biology, National University of Singapore, 10 Kent Ridge Crescent, Singapore 0511

† Institute of Neurology, 1 Wakefield Street, London WC1N 1PJ, UK

THE Ras-related Rho subfamily of GTP-binding proteins (p21s), which includes Rho, Rac and Cdc42Hs, is implicated in different aspects of cytoskeletal organization^{1,2}. These proteins behave like Ras (p21^{ras}) in that their active GTP-bound form is inactivated by intrinsic hydrolysis of the nucleotide γ -phosphate, which can be stimulated by GTPase-activating proteins (GAPs). We have previously shown that there is a diversity of GAPs that recognize this subfamily³, including *n*-chimaerin, which is enriched in the hippocampus⁴; we also detected proteins that bind these p21 proteins and seem to inhibit GTP hydrolysis. We now report the characterization of a hippocampal complementary DNA encoding a tyrosine kinase that specifically binds Cdc42Hs in its GTP-bound form. This binding is mediated by a unique sequence of 47 amino acids C-terminal to an SH3 domain and inhibits both the intrinsic and GAP-stimulated GTPase activity of Cdc42Hs. Our findings indicate that there may be a regulatory mechanism that sustains the GTP-bound active form of Cdc42Hs and which is directly linked to a tyrosine phosphorylation pathway.

Of the non-GAP proteins that interact with GTP-p21s, the most prominent in the hippocampus are the Rac1-binding and Cdc42Hs-binding proteins of *M_r* 60–66K (Fig. 1a). Screening of a human hippocampal expression library with [γ -³²P]GTP-Cdc42Hs (which gives the strongest signal in tissue extracts) yielded a positive clone: extracts of isopropylthiogalactoside (IPTG)-induced *Escherichia coli* harbouring the plasmid derivative of this phage contained a protein of *M_r* 45K which bound Cdc42Hs but not Rac or Rho (Fig. 1a). The sequence of the 2.2-kilobase (kb) cDNA contained an open reading frame of 379 amino-acid residues contiguous with the vector corresponding to the size of the expressed protein in Fig. 1a. A low-level messenger RNA (~5 kb) from human tissues was detected with a DNA probe from the coding region (data not shown). The

sequence of a full-length 4.6-kb cDNA from the same library showed that the original cDNA encoded a protein truncated at both N and C termini (Fig. 2a). The expressed truncated protein (termed tACK; see later) and its derivatives were used in subsequent experiments. Based on its p21 specificity and size, it is unlikely that the Cdc42Hs-binding protein corresponds to the hippocampal 60–66K Cdc42Hs/Rac1-binding proteins; expression screening thus enabled us to characterize a protein of lower abundance than these. The first 270 residues of tACK align with the catalytic domain of protein tyrosine kinases, and the critical residues in all 11 conserved regions are retained⁵ (Fig. 2c, asterisks). The region N-terminal to the kinase domain does not contain a transmembrane motif. Comparison of the full-length 1,091-amino-acid protein with other tyrosine kinases suggests that this activated Cdc42Hs-associated kinase (ACK) is not closely related to any of these, and represents a separate class. ACK has some similarity to the focal adhesion kinase⁶: a methionine is present in block IX (Fig. 2c), whereas an A/S residue is present at the same position in all other kinase domains, and a region of ~450 residues at the C terminus of both is rich in proline. Here, ACK shares homology with the 50K protein termed a highly inducible transcript termed gene 33 (ref. 7). When expressed as a soluble glutathione-S-transferase (GST) fusion protein (Fig. 1b, lane 2), the kinase was able to phosphorylate a synthetic polymer containing tyrosine, but could not autophosphorylate itself (Fig. 2d). As well as showing optimal alignment with the *c-src* product in the catalytic region, ACK contains a Src-homology 3 domain (SH3); this 50-residue motif is widely distributed⁸ and is present in several other non-receptor kinases. Figure 2b shows alignment to recognized SH3 motifs: it has been suggested that the observed sequence diversity allows binding of the SH3 domain to a variety of targets⁹.

Analysis of purified GST-fusion proteins containing different regions of tACK (Fig. 1b) indicated the SH3 motif was not required for binding of GTP-Cdc42Hs. Although tACK (1–374) (lane 2) does bind Cdc42Hs, tACK (1–344), which contains the SH3 domain, does not (lane 3). A region C-terminal to SH3, comprising 52 residues (327–379) fused to GST, was sufficient (Fig. 1b, lane 5). When this region from the full-length clone and the sequence C-terminal to it was expressed, the fusion protein also bound Cdc42Hs (not shown), suggesting that it is functionally independent. The sequence responsible for binding to Cdc42Hs is different from any described previously and much smaller than the GAP domains.

To determine whether binding to tACK (327–379) required

the GTP- or GDP-bound form, Cdc42Hs (cleaved from the GST-fusion protein) containing different radiolabelled guanine nucleotides was passed down a column containing excess immobilized GST/tACK (327–379) protein. [γ - 32 P]GTP-Cdc42Hs is completely retained on this but not the control (GST) column, and is co-eluted with the binding domain on addition of glutathione (Fig. 3a). To investigate the interaction of GDP-p21, we used γ - and α -labelled GTP bound to Cdc42Hs, and allowed 50 per cent GTP hydrolysis before loading onto identical ACK columns (Fig. 3b). The first peak corresponding to [α - 32 P]GDP-Cdc42Hs is only slightly retarded compared with the flow-through shown in Fig. 3a, indicating that binding is only very weak. The glutathione elution pattern of [α - 32 P]GTP-Cdc42Hs was identical to that [γ - 32 P]Cdc42Hs, confirming that ACK binds preferentially to Cdc42Hs in its GTP-bound form.

It has been proposed in the case of Ras that variable conformations of the polypeptide loops involved in GTP binding may be

partly responsible for the low intrinsic rate of hydrolysis¹⁰. Tight binding to these loops could attenuate GTP hydrolysis by restricting the available conformations. tACK (1–375) and tACK (327–379) act as GTPase inhibitors towards Cdc42Hs, but not towards Rac, which does not bind ACK (Fig. 4a). At room temperature the presence of an equimolar amount or excess of binding domain increases the half-life of GTP bound to Cdc4Hs from 6.5 to 10 minutes. This is not explained by an alteration in GTP-exchange rate, which is much slower ($t_{1/2}$ ~45 min). This GTPase inhibition is not as dramatic as the stimulation elicited by GAPs (which, for example, is ~100-fold for Rac1 GTPase with stoichiometric amounts of chimaerin³); however, the binding domain can also severely impair GTPase activation by the Bcr protein GAP domain (Fig. 4b). Thus with equal amounts (1 μ g) of BCR, ACK binding domain, and Cdc42Hs, the GAP is ineffective (compare with Cdc42Hs alone). Figure 4b shows that a 50-fold higher concentration of BCR is required to elicit the same stimulation of Cdc42Hs GTPase in the presence

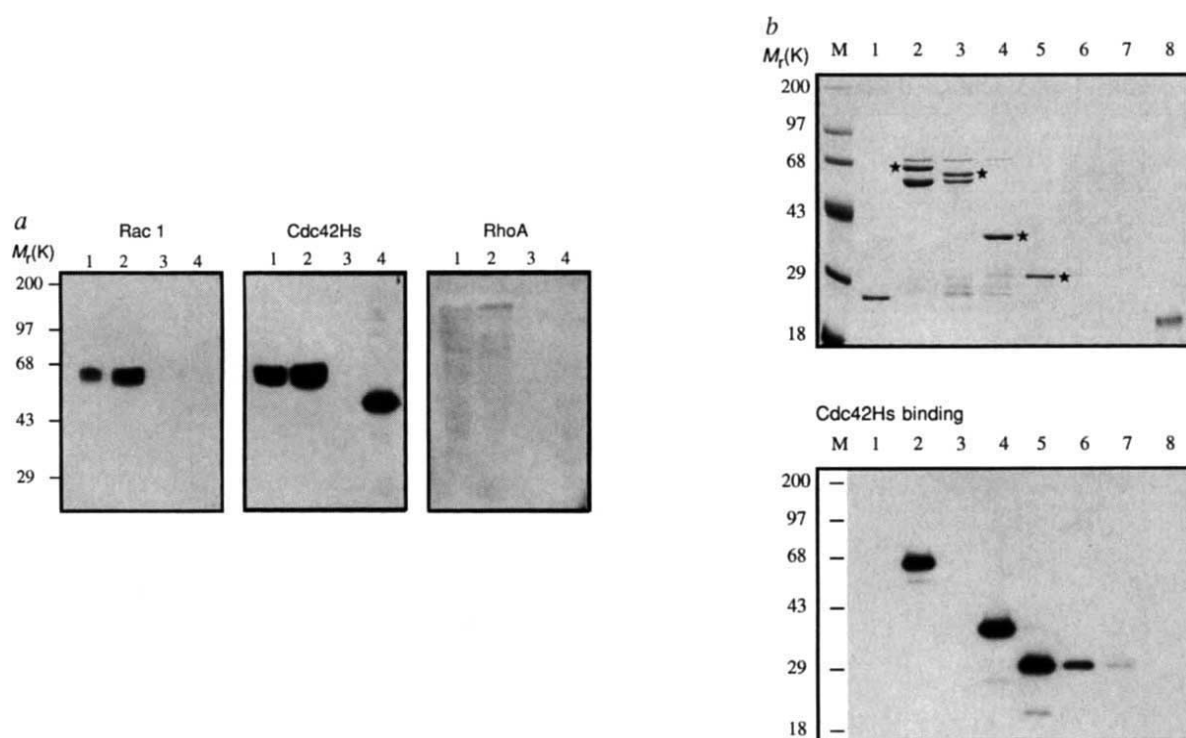


FIG. 1 *a*, Association of p21s with recombinant ACK protein. Identical western blots showing binding proteins present in a rat hippocampal detergent extract (200 μ g; lane 1), and soluble proteins (S-100 proteins; 200 μ g, lane 2). Total sonicated lysates from IPTG-induced control *E. coli* cells (lane 3) and those containing tACK bluescript plasmid (lane 4) were separated by SDS-PAGE (ref. 3), and transferred to nitrocellulose. These were probed with [γ - 32 P]GTP-GST/p21 for 5 min at room temperature, washed and autoradiographed at -20°C overnight. *b*, Localization of the Cdc42Hs binding domain. Top panel, Coomassie blue-stained gel. GST-fusion proteins are marked with an asterisk to differentiate them from copurifying *E. coli* stress proteins and proteolytic breakdown products. Bottom panel, Cdc42Hs binding. Lane M, migration of M_r standards. Lanes 1, glutathione S-transferase (26K); when fused to tACK residues 1–374, the expected fusion of 66K is seen (4 μ g; lanes 2). Deletion of 35 residues from the C terminus (tACK (1–344); lanes 3) leads to loss of Cdc42Hs binding. From the band intensities it can be seen that fusions containing the SH3 domain and C terminus, residues 263–374 (3 μ g; lanes 4) or 327–379 (2 μ g; lanes 5), bind as efficiently as the full construct. The smallest construct, tACK (327–379) was used in most subsequent experiments because the purified protein contained no other bands: at a loading of 200 ng (lanes 6) or 20 ng (lanes 7) the protein is easily detected by Cdc42Hs binding. Cleaved Cdc42Hs used in subsequent experiments is shown in lane 8.

METHODS. A human hippocampal λ ZAP library (Stratagene) was plated out

at a density of 5×10^4 PFU per 25-cm plate: phage were grown at 42°C until visible then induced by overlaying a nitrocellulose filter soaked in 10 mM IPTG for 2 h at 37°C and at room temperature overnight. The filter was removed and blocked for 1 h in 'renature' buffer (PBS containing 1% bovine serum albumin, 0.5 mM MgCl_2 , 50 μM ZnCl_2 , 0.1% Triton X-100 and 5 mM DTT). Filters (20×20 cm) were probed with [γ - 32 P]GTP-labelled GST-Cdc42Hs solution and washed as for the western blots. From this library, a screening of 5×10^5 plaques gave one positive after secondary and tertiary screening, which was converted to the Bluescript plasmid form according to the supplier's protocol (Stratagene). The brain particulate fraction was solubilized in 0.5% deoxycholate and 0.5% Triton X-100. Proteins were separated on 10% polyacrylamide gels, transferred to nitrocellulose and incubated at 4°C in 'renature' buffer³. The recombinant GST-p21s (ref. 3) were labelled with [γ - 32 P]GTP (10 μCi ; NEN) in 50 μl of exchange buffer (50 mM NaCl, 25 mM MES, 2 mM EDTA and 0.05% Triton X-100, pH 6.5) for 5 min at 22°C , and diluted into GAP buffer³ (containing 0.5 mM GTP) to a final concentration of $1 \mu\text{g ml}^{-1}$. Filters were soaked in this buffer at 4°C , transferred to 1% agarose for 5 min and washed (3×1 min, 50 mM NaCl, 25 mM MES, 5 mM MgCl_2 , 0.05% Triton X-100, pH 6.5). Deletion constructs of tACK were made by subcloning restriction fragments inframe into pGEX 3x vector (Pharmacia): XL-1 blue cells harbouring the plasmid were induced with 0.5 mM IPTG at 25°C for 3 h and fusion proteins were purified using standard protocol.

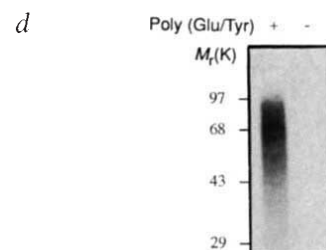
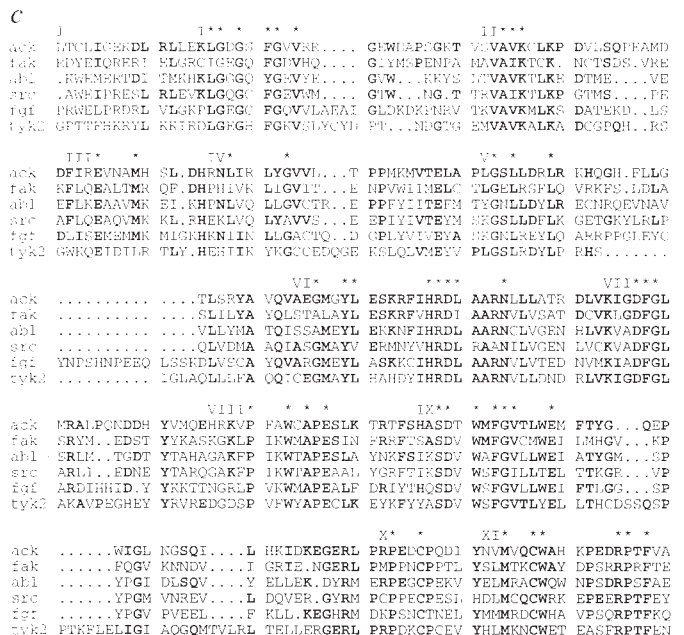
of ACK. *In vivo* this class of inhibitory proteins may counteract the effect of the many GAPs for Cdc42Hs (ref. 3). We have characterized a number of such cytosolic proteins, including those shown in Fig. 1a, which have similar binding and GTPase inhibitory properties to ACK, although their p21 selectivity varies (E.M., T.L., H.S. and L.L., manuscript in preparation).

The molecular components directly regulated by activated p21 proteins have remained largely elusive, but they must be

preferentially associated with the GTP form of the protein. GAPs downregulate active GTP-bound p21s by stimulating intrinsic GTP hydrolysis¹¹ (although p120 Ras-GAP itself has effector functions¹², requiring the flanking SH3 domain¹³), with nucleotide-exchange factors regenerating the active GTP-bound from the GDP-bound p21s. For Cdc42Hs, ACK and Cdc42GAPs may have opposing regulatory mechanisms: a protein that counteracts the effects of p120 Ras-GAP on Ras GTPase has been



FIG. 2 Amino-acid sequence of the Cdc42Hs-associated kinase. **a**, The open reading frame corresponding to the full-length clone ACK-3 is shown, with residues of tACK given in bold (numbered to the right). The C terminus of tACK contains six residues (GIPIGP) in addition to these. The open reading frames predict a polypeptide of 43K for tACK and 120K for the full-length ACK, which corresponds to the observed size of *in vitro*-translated RNA derived from ACK-3 cDNA (not shown). The position of the SH3 motif is underlined, and the Cdc42Hs binding domain is indicated. **b**, Alignment of tACK 279–327 with other SH3 motifs. Residues (and conservative changes) found predominantly at a given position are shown in uppercase; the five regions of greatest similarity are in bold. Spaces (–) are introduced for optimal alignment. Abbreviations refer to: the products of the cellular proto-oncogenes *src* and *vav*; *Acanthamoeba* myosin-1B, MYO-1B; yeast budding related proteins BEM1 and actin-binding protein (ABP); and the p85 β -subunit of phosphatidylinositol-3-OH kinase, which has an intervening 17-residue insert (marked). Sequences are as in ref. 6. Amino (n) or carboxyl (c) refer to the position of the SH3 in proteins that contain more than one motif. **c**, Comparison of ACK kinase domain with other protein tyrosine kinases. Sequences from those tyrosine kinases (of different classes) showing the highest amino-acid identity to ACK were aligned with the aid of the program 'pileup' (UWGGC, ref. 22). In the designated blocks I–XI for protein kinases⁵, the highly conserved residues (accounting for conservative substitutions L/I/V, G/A and Y/F) among tyrosine kinases are marked with asterisks. Amino acids conserved between ACK and others are shown in



d, Tyrosine kinase activity of ACK. The GST-tACK 1–374 (2 μ g per 100 μ l in kinase buffer, 50 mM HEPES, 5 mM $MgCl_2$, 5 mM $MnCl_2$, pH 7) was incubated with or without 10 μ g poly(Glu/Tyr) (4:1; M_r ~60,000; Sigma) in the presence of 10 μ Ci [γ -³²P]ATP (Amersham) for 5 min at 30 °C. Aliquots were boiled in SDS buffer then run on a 10% SDS-polyacrylamide gel, fixed, dried and placed on Hyperfilm MP overnight. In the absence of kinase no label was transferred to the poly(Glu/Tyr) (not shown).

METHODS. Five λ ZAP cDNA clones were obtained by DNA hybridization screening of the human hippocampal library with the 5' (coding sequence) 1.1-kb *Eco*R1 fragment of tACK. Of these, the longest clone ACK-3 (4.6 kb extending from a polyadenylation site) and the 2.2-kb tACK cDNAs were fully sequenced in both strands using restriction subclones and oligonucleotides complementary to internal sequences. The initiation codon for ACK-3 is the 5'-most ATG in the correct frame at position 451 in the cDNA. Two shorter clones containing the sequence encoding the kinase, SH3 and Cdc42Hs-binding domain were identical to ACK-3 in the region 3' to these, which is different from tACK. The point of sequence divergence is bordered by an internal *Eco*R1 site in the tACK cDNA at a position close to the end of the tACK open reading frame, indicating that the original clone was truncated at the 3' end and spliced to an unrelated cDNA. The Genbank accession number of ACK cDNA is L13738.

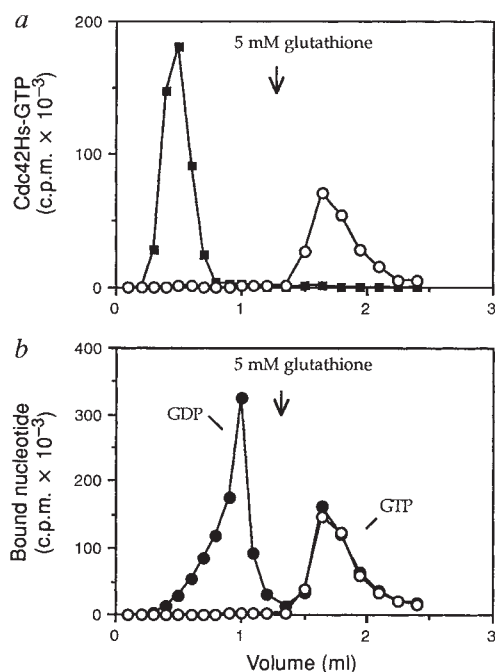


FIG. 3 Nucleotide dependence of Cdc42Hs binding. *a*, Columns (0.25 ml) containing 10 mg ml⁻¹ immobilized GST (■) or 6.5 mg ml⁻¹ GST-tACK 327-274 (○) were loaded with 2 μ g [γ -³²P]GTP-labelled Cdc42Hs in column buffer containing 0.5 mM GTP at 8 °C. At the point marked by the arrow the buffer was switched to one containing 5 mM glutathione, which elutes the fusion protein. Fractions were collected on ice, and subsequently analysed by dotting 20 μ l onto nitrocellulose and washing (as for Fig. 1) to remove free nucleotide and phosphate. *b*, Cdc42Hs (2 μ g) labelled either with [α -³²P]GTP (●) or [γ -³²P]GTP (○) was incubated in column buffer for 5 min at room temperature to allow ~50% hydrolysis (see Fig. 4 legend) and passed down identical GST-tACK columns as in *a*. Fractions were analysed for bound nucleotide as already described.

METHODS. GST-Cdc42Hs was purified and cleaved *in situ* on glutathione-Sepharose (Pharmacia) in the presence of 10 units per ml thrombin (Sigma) and 1 mM CaCl₂ for 1 h at room temperature; the released Cdc42Hs (Fig. 1b, lane 8) was collected and stored at -70 °C with 5% glycerol. Column buffer contained 50 mM NaCl, 25 mM MES, pH 6.5, 1 mM MgCl₂ and 0.05% Triton X-100. Nitrocellulose filters were washed in the same buffer containing 5 mM MgCl₂.

reported¹⁴, and the 28K GDP-dissociation inhibitor protein inhibits both the intrinsic and GAP-stimulated GTPase of Cdc42Hs (ref. 15). Mammalian Cdc42Hs is able to complement lethal loss of Cdc42 in yeast¹⁶ (overexpression of Cdc42Sc leads to random budding) and is potentially linked to mammalian cellular transformation through the *dbl* oncogene, which encodes a nucleotide-exchange factor for Cdc42Hs but not Ras (ref. 17). A Cdc42Hs-associated tyrosine kinase might be recruited for such a process. Growth factor stimulation leads to tyrosine kinase-mediated activation of both Ras and the Rho subfamily p21 proteins¹⁸ and perhaps phosphorylation of Cdc42Hs (ref. 19). Ras is in turn implicated in the regulation of downstream protein kinases such as Raf²⁰, and our findings should therefore provoke a search for kinases that might interact directly with Ras. We have previously shown that the GTP forms of Rho, Rac and Cdc42Hs have a number of targets, including GAPs, in different tissues³. ACK therefore represents a new class of proteins that affect the Rho subfamily and which oppose the activity of GAPs. □

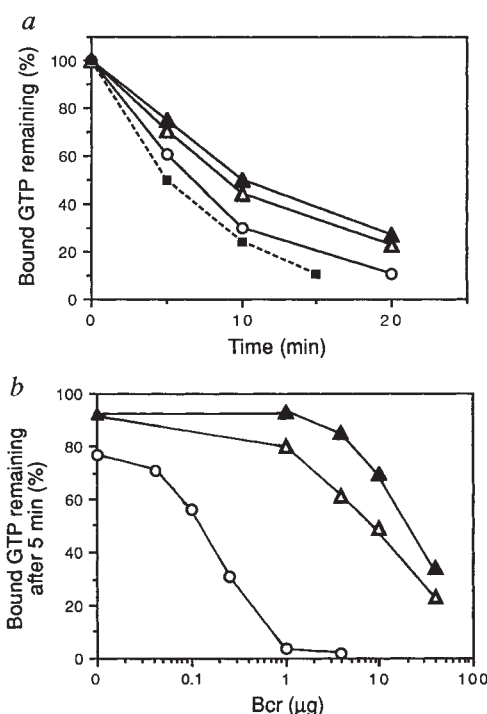


FIG. 4 *a* Effect of tACK (327-379) binding upon GTP hydrolysis by Cdc42Hs. The intrinsic hydrolysis rate at 25 °C was determined from the amount of [γ -³²P]GTP-Cdc42Hs remaining (○) with time; this rate is attenuated in the presence of 1 μ g per μ g p21 (~equimolar; triangles) or 10 μ g ACK fusion protein (▲); 10 μ g TACK (1-374) gave the same inhibition (not shown). Rac1 intrinsic GTP hydrolysis is unaffected (■; with and without 10 μ g TACK (327-379)). At different times, samples were collected simultaneously; each data point represents the average of two measurements. *b*, The presence of ACK opposes the GAP activity of Bcr (the product of the *bcr* (breakpoint cluster region) gene)²¹. In the absence of ACK, Bcr causes the concentration-dependent decrease in GTP bound to Cdc42Hs shown (○). With GST-tACK (327-379), this GAP activity is markedly reduced. The ACK fusion protein was preincubated with 1 μ g [γ -³²P]GTP-Cdc42Hs for 5 min on ice, then Bcr (per μ g Cdc42Hs) was added and the sample incubated at 20 °C. After 5 min, aliquots were assayed for bound radionucleotide as for Fig. 3. **METHODS.** For solution assays, 10 μ l labelled Cdc42Hs were diluted at 4 °C into 100 μ l final volume of 'GAP' buffer³ containing the appropriate amount of tACK (327-379), incubated for 5 min, then exposed to the reaction temperature. At the times indicated, 15 μ l was dotted onto nitrocellulose, washed at 4 °C and counted. DNA corresponding to Bcr residues, 1,059-1,271 and comprising only the GAP domain²¹ was cloned from a human cDNA into pGEX-2T and transformed into the *E. coli* XL-1 cells. IPTG-induced cells yielded the GST-Bcr fusion protein which on SDS-polyacrylamide gels showed a single band corresponding to *M*_r 45K, which was used without further treatment.

- Manser, E. *et al.* *J. biol. Chem.* **267**, 16025-16028 (1992).
- Hall, C. *et al.* *J. molec. Biol.* **211**, 11-16 (1990).
- Hanks, S. K., Quinn, A. M. & Hunter, T. *Science* **241**, 42-52 (1988).
- Schaller, M. D. *et al.* *Proc. natn. Acad. Sci. U.S.A.* **89**, 5192-5196 (1992).
- Lee, K.-L., Makkinje, A., Ch'ang, L.-Y. & Kenney, F. T. *Arch. Biochem. Biophys.* **269**, 106-113 (1989).
- Musacchio, A., Gibson, T., Lehto, V.-P. & Saraste, M. *FEBS Letts* **307**, 55-61 (1992).
- Cicchetti, P., Mayer, B. J., Thiel, G. & Baltimore, D. *Science* **257**, 803-806 (1992).
- Bourne, H. R., Sanders, D. A. & McCormick, F. *Nature* **349**, 117-127 (1991).
- Bollag, G. & McCormick, F. *A. Rev. Cell Biol.* **7**, 601-632 (1991).
- Martin, G. A. *et al.* *Science* **255**, 192-194 (1992).
- Duchesne, M. *et al.* *Science* **259**, 525-528 (1993).
- Tsai, M.-H., Yu, C.-L. & Stacey, D. W. *Science* **250**, 982-985 (1990).
- Hart, M. J. *et al.* *Science* **258**, 812-815 (1992).
- Shinjo, K. *et al.* *Proc. natn. Acad. Sci. U.S.A.* **87**, 9853-9857 (1990).
- Hart, M. J., Eva, A., Evans, T., Aaronson, A. & Cerione, R. A. *Nature* **354**, 311-314 (1991).
- Ridley, A. J. & Hall, A. *Cell* **70**, 389-399 (1992).
- Hart, M. J., Polakis, P. G., Evans, T. & Cerione, R. A. *J. biol. Chem.* **265**, 590-601 (1990).
- Dickson, B., Sprenger, F., Morrison, D. & Hafen, E. *Nature* **360**, 600-603 (1992).
- Diekmann, D. *et al.* *Nature* **351**, 400-402 (1991).
- Devereux, J., Haeblerli, P. & Smithies, O. *Nucleic Acids Res.* **12**, 387-395 (1984).

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- Hall, A. *Molec. Biol. Cell* **3**, 475-479 (1992).
- Drubin, D. G. *Cell* **65**, 1093-1096 (1991).

Fission yeast *chk1* protein kinase links the *rad* checkpoint pathway to *cdc2*

Nancy Walworth, Scott Davey & David Beach*

Howard Hughes Medical Institute, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, 11724, USA

THE dependence of cell-cycle progression on the integrity of the genome has been described as checkpoint control^{1,2}. A number of mutants of the fission yeast *Schizosaccharomyces pombe*, selected for their sensitivity to DNA damage caused by radiation (*rad* mutants) or to the DNA synthesis inhibitor hydroxyurea (*hus* mutants) have been classified as checkpoint mutants because they fail to arrest the cell cycle in response to DNA damage or incompletely replicated DNA³⁻⁶. Coupling of the checkpoint pathways that monitor DNA repair and replication to control of the cell cycle is essential. In a search for components that interact with the cell-cycle regulatory kinase p34^{cdc2}, we have identified a novel fission yeast protein kinase homologue which is involved in cell-cycle arrest when DNA damage has occurred or when unligated DNA is present. We have called the gene encoding this protein *chk1* for checkpoint kinase. Multiple copies of *chk1* partially rescue the ultraviolet sensitivity of *rad1-1*, a mutant deficient in checkpoint control³⁻⁵. Identification of a gene involved in checkpoint control as a rescue of a *cdc2* mutant links the *rad1*-dependent DNA-damage-sensing pathway and p34^{cdc2} activity.

In *S. pombe*, entry into mitosis is regulated by p34^{cdc2} (refs 7, 8) which is activated by the *cdc25*-encoded tyrosine phosphatase⁹⁻¹² and inhibited by the *mik1*- and *wee1*-encoded protein kinases¹³⁻¹⁵. In a search for additional elements that might regulate p34^{cdc2} function, we introduced a multicopy gene bank into a cold-sensitive *cdc2* mutant, *cdc2-r4* (ref. 14), and isolated plasmids that allowed colony formation at 18 °C. One of these plasmids was subcloned and sequenced (Fig. 1a, b). The inferred *chk1* protein sequence is 30-40% identical to the family of serine/threonine protein kinases (Fig. 1a)¹⁶. Disruption of the kinase catalytic domain (*chk1::ura4*) results in viable haploid cells (Fig. 3a).

A *chk1* disruptant displays phenotypes characteristic of mutants defective for checkpoint control⁴⁻⁶. The *chk1::ura4* mutant exhibits sensitivity to ultraviolet radiation intermediate between *rad1-1* and wild-type cells (Fig. 2a), suggesting that *chk1* is not solely responsible for damage-induced arrest. When a temperature-sensitive *cdc25-22* strain is arrested at the G2-M border, irradiated with ultraviolet, then shifted to permissive temperature, the cells display an ultraviolet-dose-dependent delay in the onset of mitosis⁵. A *chk1::ura4 cdc25-22* strain enters mitosis when the *cdc25-22* block is released at doses where *cdc25-22* remains arrested (not shown). The ultraviolet sensitivity of *chk1::ura4* can be largely rescued by the imposition of a G2 delay (Fig. 2b), suggesting that the *chk1* protein plays no part in the repair process^{4,5,17}.

The absence of *chk1* or checkpoint *rad* function^{4,5} allows cells to bypass arrest normally induced by unligated DNA. At 35.5 °C, a strain harbouring a mutant allele of the DNA ligase gene (*cdc17-K42*) arrests in late S phase and elongates¹⁸, slowly losing viability over time. However, a *chk1::ura4 cdc17-K42* double mutant loses viability quickly at 35.5 °C (Fig. 2c). DNA staining reveals that although *cdc17-K42* cells arrest in interphase, the *chk1::ura4 cdc17-K42* strain attempts to enter mitosis, resulting in a variety of chromosomal abnormalities (not shown).

Inhibition of DNA synthesis by hydroxyurea (HU) causes checkpoint *rad* or *hus* mutants to lose viability as they enter

mitosis in the presence of unreplicated DNA⁴⁻⁶. The *chk1::ura4* strain loses viability very slightly if compared to wild-type cells, but is not nearly as sensitive to HU as *rad1-1* (Fig. 2d). Furthermore, although the septation index for wild-type or *chk1::ura4* cells drops to zero, an indication of cell-cycle arrest, the septation index of *rad1-1* cells in HU does not drop⁴ (not shown). The behaviour of *chk1::ura4* in HU distinguishes it from the other checkpoint *rad* and *hus* mutants⁴⁻⁶.

Like *wee1-50* double mutants with the *rad* checkpoint genes^{4,5}, *chk1::ura4 wee1-50* fails to form colonies at 36 °C and appears to enter mitosis prematurely (Fig. 3). When grown in liquid culture and shifted to 36 °C, *chk1::ura4 wee1-50* cells divide at a very small size, lay down septa improperly and undergo multiple rounds of nuclear division before completing cell separation. Although *chk1* function is not normally required for vegetative growth, it is required as a mitotic inhibitor in cells lacking a functional *wee1* product in which G2 is shortened. Consistent with this observation, constitutive overexpression of *chk1* in wild-type cells results in mitotic delay, as indicated by slight cell elongation (not shown). Lethal entry into mitosis of *chk1::ura4 wee1-50* can be rescued by *cdc2-r4*; the triple mutant *cdc2-r4 chk1::ura4 wee1-50* forms colonies of wee cells at high temperature (not shown). The isolation of an apparent mitotic inhibitor, *chk1*, as a multicopy suppressor of a mutant that is delayed in the cell cycle, *cdc2-r4*, is difficult although not impossible to reconcile, given that p34^{cdc2} is subject to multiple levels of regulation. Further characterization of the *cdc2-r4* strain will be required to understand the interaction between p34^{cdc2} and *chk1*.

The phenotype of *chk1::ura4 wee1-50* is similar to that of *rad1-1 wee1-50* (refs 4, 5); therefore, we tested whether *chk1* might interact with *rad1*. A multicopy *chk1*-containing plasmid was introduced into *rad1-1 wee1-50* and rescued the temperature-sensitive lethality of this strain (not shown). Overexpression of *chk1* partially rescues the ultraviolet sensitivity of *rad1-1* (Fig. 4). Although a 2-hour G2 delay caused by *cdc25-22* arrest also rescues *rad1-1* (ref. 5), any delay caused by overexpression of *chk1* cannot be equivalent because the doubling time of the strain is not increased by 2 hours. Furthermore,

FIG. 1 *chk1* encodes a protein kinase. a, Nucleotide and predicted amino-acid sequence of *chk1*. Shown in lower-case type are seven introns determined by sequence comparison of the genomic and cDNA clones. The 5' donor and 3' acceptor splice sites are underlined, as well as the putative branch points. Removal of the first 6 introns results in an open reading frame (ORF) of 496 amino acids (predicted M_r 56,469). The seventh intron appears to be in the 3' untranslated region. Amino acids highly conserved in the catalytic domains of protein kinases are highlighted in boxes¹⁶. b, Restriction map showing select enzymes. H, *HindIII*; N, *NdeI*; A, *AflIII*; Bg, *BglIII*; B, *BamHI*; R, *EcoRI*; P, *PstI*. Small boxes represent introns. The ORF is represented by the arrow with ATG (start) codon and STOP codons as indicated. Deletions used to delineate the rescuing activity are shown. An ORF on the opposite strand is completely removed in pCHK1d2 and pCHK1d3, yet the plasmids, like pCHK1d1, still rescue *cdc2-r4*. c, Schematic of construct used to disrupt *chk1*.

METHODS. Standard genetic and molecular techniques were used¹⁴. An 11-kb insert isolated from an *S. pombe* genomic library allowed SP1181 (*h^{-S} cdc2-r4 leu1-32*) to grow at 18 °C. A 3.4 kb *HindIII* fragment sub-clone which rescued was sequenced. Protein sequence comparisons to the GenBank database were done using FASTA¹⁹. cDNA isolates were obtained from an *S. pombe* library generated from poly(A)⁺ RNA in λ-zap (Stratagene) using a *HindIII*-*BamHI* probe from the 5' end. To disrupt *chk1*, the *AflIII*-*BglIII* fragment encoding the entire catalytic domain (with the exception of the first two glycine residues) plus the subsequent 40 amino acids were removed and replaced with the *ura4* gene. The *HindIII* fragment with the disrupted gene was introduced into SP826 (*h⁺/h⁺ ura4-D18/ura4-D18 leu1-32/leu1-32 ade6-210/ade-216*). Stable Ura⁺ transformants were selected and disruptants confirmed by Southern blot analysis. h⁹⁰ diploids were selected and sporulated. The plasmids pCHK1d1 and d2 were generated by exonuclease digestion. pCHK1d3 was created by deleting from the *BamHI* site within the fragment to a *BamHI* site in the pSP1 plasmid (ref. 20).

* To whom correspondence should be addressed.

expression of *chk1* from its own promoter on a multicopy plasmid also partially rescues *rad1-1*, but causes no detectable cell-cycle delay (Fig. 4). These results imply that there may be some overlap between the pathways on which *rad1* and *chk1* function to regulate *p34^{cdc2}*.

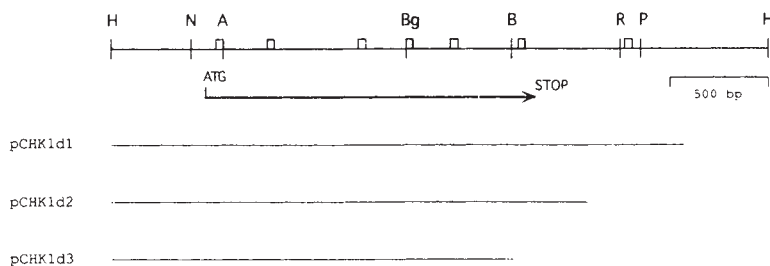
The phenotypes associated with *chk1::ura4* are most similar

to mutants of *rad21* of *S. pombe* or *RAD9* of *Saccharomyces cerevisiae*. These strains fail to arrest the cell cycle in response to DNA damage or unligated DNA^{5,17}, but do arrest in the presence of the DNA synthesis inhibitor HU. In contrast to *rad21* (ref. 5), however, a *chk1::ura4 weel-50* double mutant is inviable at high temperature. As a protein kinase, *chk1* is

a

hindIII
1 AAGCTTATTACAAACATTAACTCATTATTCGCTTCTCAATCTACAAATGCATTAGAG
61 ACTTTTCTCTAAACACTGTTTATTTTACCGTTTGTAAACGCTATGACCGTCAACAGC
121 CATGCCAGCAGAAGAGAGTTTATAGAATTAGTATGAGGGTCTGCTTTGCATATCAGA
181 ATCCATTCTCAAGTTGTTACTGTAGTTTCTCAATGAGTACGTGGTGTAGGAGAGTAAG
241 TTATAAACACACACACATGTGCTAATGTACACAGTAACGAAACCGGATATCAAAAT
301 TATAGTCCAATTTTATATCATATATATAGATTTAAACCTCATTTAAAAATTACCTTAATTT
361 AATCTTTCCAGTCTTTGATGATGTTAAGACTGTAATAATACACATGCCAAAATTGCA
421 ACATATGCTGTGTTATATATATCTCTCTTCCCTTACACCAAAAGATGGCTCAAAA
481 TTAGATAACTTCTTACCATTATGTTAGAGAAATAGTACTGGGGCTTTTGGCTGctat
L D N F P Y H I G R E I [Q] T [Q] A F A S
541 gaattttgattgaaataactatataactaacttaagcaagCGTCCGTTTATGTTACGAT
[AFIII] [IV] R L C Y D
601 GATAATGCTAAATATATGCTGCTCAATTTGTCAATAAAAAACATGCTACTAGTTGTATG
D N A K I Y A V [E] F V N K K H A T S C M
661 AATGCAAGGGTATGGGCTAGAAGGATGGCTAGTGAATACAACCTCACAACTATGCAAT
N A G V W A R R M A S [E] I Q L H K L C N
721 GGACATAAAATATCATCTTTTATAATACAGCAGAAATCCCAATGGAGATGGGTA
G H K N I I H F Y N T A E N P Q W R W V
781 GTACTTGAATTTGCTCAAGGTGGTACTTATTGACAAAATAGTgggtgcatgaacctt
V L E F A Q G G D L F D K I G
841 tcgcttttactaacggacttacaatttagAGCCTGATGATGATGAGGACGTCGC
P D V G I D E D V A
901 TCAGTTTATTTTGTCTCAACTTATGAAGGATTTCTCTCATGCACTTAAAGGTGTCGC
Q F Y F A Q L M E G I S F M H S K G V A
961 GCATCGAGACTTGAACCTGAGAACCTCTTTAGATTACAATGGTAACTTAAAGATTTC
H R [D] L [E] P E [E] I L L D Y N G N L K I S
1021 AGACTTCGGCTTTGCATCTTTATTTTCTATAAAGGTAAAGTAGCTTTTGAATAGTCC
[D] F [Q] F A S L F S Y K G K S R L L N S P
1081 AVTGGGTAGTCCACCATACGCTGCTCCAGAAATTACACAGCAGTATGTTGTTCAAAAGT
V G S P P Y A A [D] [E] I T Q Q Y D G S K V
1141 TGATGCTGGTCTCGGTATATTTCTTTTGTGTTTATTAGGCAACACCTTGGGA
[D] V W S C [Q] I I L F A L L L G N T P W D
1201 TGAAGCAATTAGCAACACTGGTACTTGTCTTATAAAAAAATGTGAAGCCCGTC
E A I S N T G D Y L L Y K K C E R P S
1261 TTACCACCATGGAACCTGTTGCTCCAGGAGCCTATTglaagtctgactgaaattcct
Y H P W N L L S P G A Y S
1321 ttaagctaatgatcagCGATTATACCCGGAATGCTTCAAGTGATCCATTTAAGCGCTAC
I I T G M L R S D P F K [E] Y
1381 TCTGTCAACATGTTGTACAGCACCATGCTTACCTCAAGTACACATTTTCAACTAAA
S V K H V V Q H P W L T S S T P F R T K
1441 AATGGGAATTGTGCCATCCTGTAGCATTGGCATCAAGGCTCATGTTGAACTTCGTATT
N G N C A D P V A L A S R L M L K L R I
1501 GATTTAGATAAACCGCTTGGCTCATCTAGAGCATCTCAAAAGtaagactcttgacacag
D L D K P R L A S S R A S Q N
1561 ttgtttcatatcttaagctctttcaattagCGATAGCGGTTTTCAATGACACAGCCAGC
D S G F S M T Q P A
1621 TTTTAAAAAATGACCAAAAGGAGTTGGATCGTGTAGAAGTTTATGGCGCTTATCCCA
F K K N D Q K E L D R V E V Y G A L S Q
1681 GCCAGTACAACCTTAATAAAATATTGACGTTACTGAAATCCTTGAAAAGACCCCTCATT
P V Q L N K N I D V T E I L E K D P S L
1741 GAGTCAGTTTGTGAGAATGAAGTTTATTAAGATTGgattattattattaaaccta
S Q F C E N E G F I K R L
1801 aattttttctactatatgaaaagGCCAAAAGGCTAAGAATTTCTACGAAATTTGTCC
A K K A K N F Y E I C P
1861 TCCTGAAAGACTCACTAGGTTTATTTCTAGGGCGCTGAGAGAAACATTATAGATCATCT
P E R L T R F Y S R A S R E V Y I D H L
1921 TTATGACAGCTACGACTACTTGAATCTCAGTGACTATGAAATATGTTGAAATCAGAC
Y D S L R L L A I S V T M K Y V R N Q T
1981 CATCTTTATGTTAATTACATGATAAAGGAAATGCTCTTTCGAAGGTGTTATTGAAC
I L Y V N L H D K R K C L L Q G V I E L
2041 AACTAATTTAGGCCATAACTTAGAGCTTATAAACTTTATAAGCGTAATGGGATCCTCT
T N L G H N L E L I N F I K R N G D P L
2101 TGAATGGAGAAAATTTTAAAGtatgctttatcatagggatttcatataactaatcoat
E W R K F F K
2161 gtgaagAACGTTGTGAGTTCAATAGGAAGCGGATTTGTTTACAGATGTTTCACAAA
N V V S S I G K P I V L T D V S Q N
2221 TTAATGCACATCTTTTGAAGGCTCGCCAGTCTGTTTGTAAAGAAACCTGTTGTTG
stop stop stop
2281 ACTTCCTGTGAATTTATTTCAATTTTGTGTAGTACAAATGATATCTAGGGAATAGG
2341 ATTATTAACGCTTTGTCAAGCAATATCAATGTTGCGAATGCTGAAGATATATTATTAG
2401 CATAAGGATAGTCTTCCGACTGCCATGCCGCTTAAATACATCAATTGACCCAATATTCT
2461 TTTCTCACAATCTTCTCTCTTTTCTCTCTTTTGTAGTATATCTGACGGCTCATTTGA
2521 TTTTAAAGGGATAACTCTTCTGAATAACTCTTTCAACAGATTCTAAAGGTCTTCATT
2581 TTCTTGGTGGAGTATAAACTCATCTCTTTTACAAAATAGTAAAGGTCTTTTGT
[EcoRI]
2641 GAATTCCTGCTCTCTTGGCTTCCAGGtaagcatgcaaggaataatgtgacatcgatga
[PstI]
2701 accagtaactatcaatgtagTTATCCATAAAATATCGCTGGAGTCTCTGACAGATTAA
2761 CATGCTTCTCTTGTAGCCTCATCTACTTTTAAAAACTCTTTCCTGGAAGTACTTCGG
2821 GAAGCTCTAAGAAACAGTTAAAGCCCTCACACAGATATGTAGCCAGCATGATTTTCAG
2881 TAACAAGTATATATCCGAAGATTCAATTAATGCATCCGGATGTCCTCTATTCTTTT
2941 CGGCTGCTACTTTGTGAACAGGTGTAGTCTCTCTAAACTCACCTTTTCATTCACTAGTC
3001 GTTCTAATGCTTTCCATAGATATGTTTGGTGAGCAGGAGATAAGATCATATAGCATTAA
3061 GCTGATGAGTTAATACCTTGATGCCATTATCCCTTTAGACAACCTTTAAGTGAAGCCTTT
3121 TAATACAGCTGTTCTGTTTACAAGTATAGCATATTTTCAAGGCAGCTATTAGTTATTT
3181 GAGGTTTCACTAATGTTTGTATGTTCCATTTTGTATATCGAAGACATTCATCAAGTTT
3241 TATCTCGTGCTCAAGATGGTAATAGTTACTGTAGTGGAAAAAGGCTTTACTCTTGA
3301 ATTGAACATCTTCGATACATATATCTAGGTTATCACAAGAAGTTTACCAAGGAATTT
3361 CAGTAGAGCACATGTCTGATCTGGGCAAAATTTGCAAAAGCTT

b



c



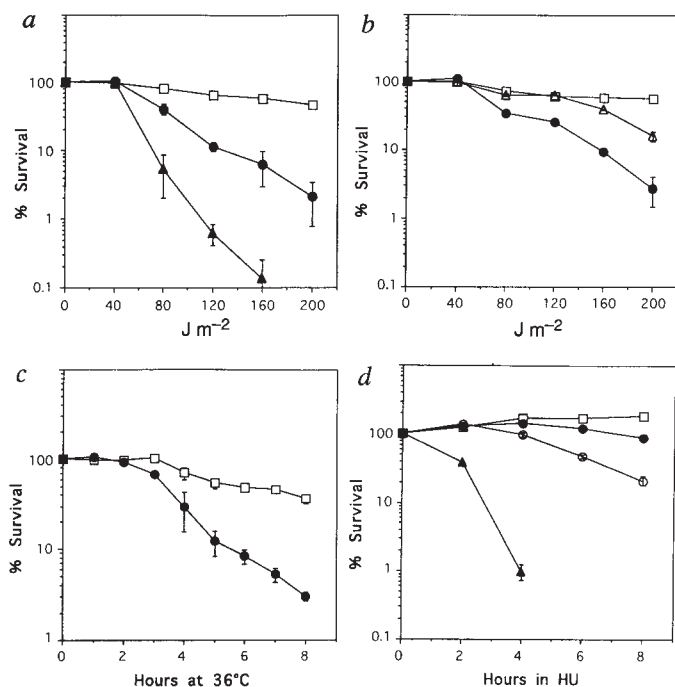


FIG. 2 Loss of *chk1* leads to sensitivity to ultraviolet irradiation or to unligated DNA. *a*, *chk1::ura4* is sensitive to exposure to ultraviolet irradiation: □, wild type (972); ●, *chk1::ura4* (SP1182); ▲, *rad1-1* (SP1187). *b*, G2 delay allows partial recovery of the ultraviolet sensitivity of *chk1::ura4*: □, wild type (972); △, *cdc25-22 chk1::ura4* (SP1184); ●, *chk1::ura4* (SP1182). *c*, *chk1::ura4* causes loss of viability in a *cdc17-K42* background: □, *cdc17-K42* (SP80); ●, *chk1::ura4 cdc17-K42* (SP1185). *d*, Response of *chk1::ura4* to transient exposure to hydroxyurea: □, wild type (972); ●, *chk1::ura4* (SP1182); ○, *cdc2-3w* (SP661); ▲, *rad1-1* (SP1187).

METHODS. Experiments were initiated with cells grown at 25 °C in liquid culture (YEA) to mid-log phase ($\sim 5 \times 10^6$ cells per ml). Survival was determined by comparing colony counts to controls after 4 days at 25 °C. Error bars indicate standard deviations. For ultraviolet sensitivity experiments (*a*, *b*), 500 cells were plated in triplicate for each dose and exposed in a UV Strata-linker (Stratagene) to fluences ranging from 40 to 200 J m⁻². In *b*, after ultraviolet exposure, plates were incubated at 36 °C for 2 h to impose the G2 delay caused by loss of function of *cdc25-22*, then moved to 25 °C. In *c*, unligated DNA was generated by incubating cells containing the temperature-sensitive *cdc17-K42* allele at 36 °C for the indicated times. To test for sensitivity to a transient exposure to hydroxyurea (*d*), mid-log phase cells were shifted into YEA containing 10 mM HU and plated in triplicate at 2-h intervals onto YEA at 25 °C as described⁴⁻⁶. Complete genotypes: 972, *h^{-S}* (wild type); SP80, *h^{-S} cdc17-K42*; SP661, *h^{-S} cdc2-3w leu1-32*; SP1182, *h^{-S} chk1::ura4 ura4-D18*; SP1184, *h^{-S} cdc25-22 chk1::ura4 ura4-D18 ade6-216*; SP1185, *h^{-S} cdc17-k42 chk1::ura4 ura4-D18*; SP1187, *h^{-S} rad1-1*.

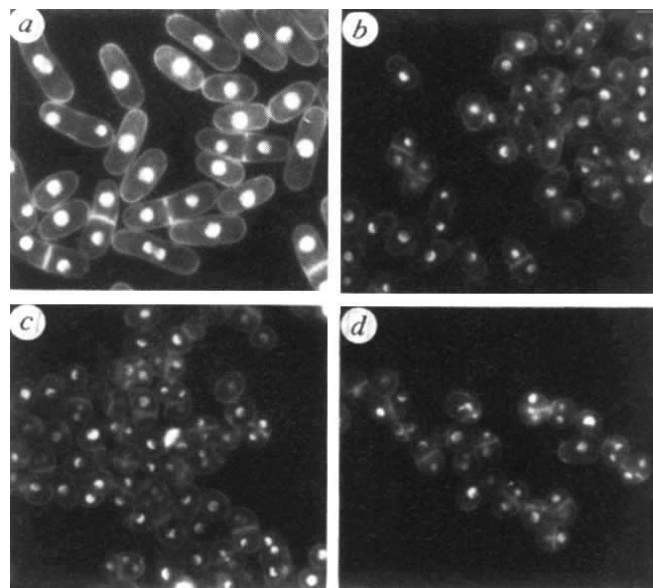


FIG. 3 A *chk1::ura4 wee1-50* double mutant is advanced in mitosis when *wee1* function is lost at 36 °C. Phenotype after 4 hours at 36 °C. *a*, *chk1::ura4* cells (SP1182, shown) are indistinguishable from wild-type cells (not shown). *b*, *wee1-50* (SP616). *c*, *chk1::ura4 wee1-50* (SP1183). *d*, *rad1-1 wee1-50* (SP1186).

METHODS. Cells were grown to mid-log phase in YEA at 25 °C, resuspended in fresh YEA at 36 °C and incubated in liquid culture for 4 h. DAPI staining was done as described with glutaraldehyde-fixed cells¹⁴. Complete genotypes: SP616, *h^{-S} wee1-50 leu1-32 ade6-210*; SP1182, *h^{-S} chk1::ura4 ura4-D18*; SP1183, *h^{-S} wee1-50 chk1::ura4 ura4-D18 ade6-216*; SP1186, *h^{-S} rad1-1 wee1-50 leu1-32*.

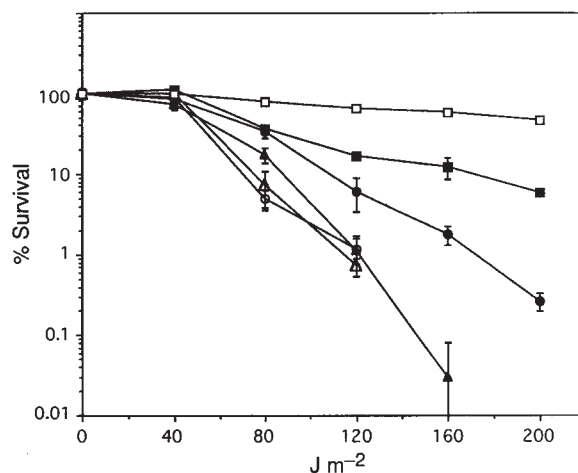


FIG. 4 Plasmids expressing *chk1* partially rescue the ultraviolet sensitivity of *rad1-1* (SP1188). ■, pADHCHK1⁺; ●, pCHK1d1; △, pCHK1d3; ▲, pADHCHK1⁻; ○, pSP1 (empty vector control); □, wild-type cells for comparison (972).

METHODS. SP1188 (*h⁺N rad1-1 leu1-32 ura4-D18*) was transformed with multi-copy, *ars1* containing plasmids. pCHK1d1 and pCHK1d3 (see Fig. 1) have the *chk1* gene under control of its own promoter. pCHK1d2 rescues to the same extent as pCHK1d1 and lacks the ORF on the opposite strand (see legend to Fig. 1). pADHCHK1⁺ and pADHCHK1⁻ have a 4.5 kb *chk1* cDNA under ADH promoter control in the correct or incorrect orientation, respectively. pSP1 is the empty vector control²⁰. Cells were grown to mid-log phase in minimal media and aliquots were plated in triplicate on media selective for the plasmids. Plates were exposed to ultraviolet light as described for Fig. 2. Plates were incubated at 25 °C and colonies counted after 5 days. Error bars indicate standard deviation of data.

likely to play a regulatory role in the link between the checkpoint pathways and cell-cycle control. The *chk1* kinase may inactivate $p34^{cdc2}$ in response to DNA damage or unligated DNA. □

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- Hartwell, L. H. & Weinert, T. A. *Science* **246**, 629–634 (1989).
- Murray, A. W. *Nature* **359**, 599–604 (1992).
- Phipps, J., Nasim, A. & Miller, D. R. *Adv. Genet.* **23**, 1–72 (1985).
- Rowley, R., Subramani, S. & Young, P. G. *EMBO J.* **11**, 1335–1342 (1992).
- Al-Khodairy, F. & Carr, A. M. *EMBO J.* **11**, 1343–1350 (1992).
- Enoch, T., Carr, A. M. & Nurse, P. *Genes Dev.* **6**, 2035–2046 (1992).
- Draetta, G. *Trends biochem. Sci.* **15**, 378–382 (1990).
- Nurse, P. *Nature* **344**, 503–508 (1990).
- Russell, P. & Nurse, P. *Cell* **45**, 145–153 (1986).

- Gould, K. L., Moreno, S., Tonks, N. K. & Nurse, P. *Science* **250**, 1573–1576 (1990).
- Dunphy, W. G. & Kumagai, A. *Cell* **67**, 189–196 (1991).
- Gautier, J., Solomon, M. J., Booher, R. N., Bazan, J. F. & Kirschner, M. W. *Cell* **67**, 197–211 (1991).
- Russell, P. & Nurse, P. *Cell* **49**, 559–567 (1987).
- Lundgren, K. et al. *Cell* **64**, 1111–1122 (1991).
- Featherstone, C. & Russell, P. *Nature* **349**, 808–811 (1991).
- Hanks, S. K. & Quinn, A. M. *Meth. Enzym.* **200**, 38–62 (1991).
- Weinert, T. A. & Hartwell, L. H. *Science* **246**, 629–634 (1989).
- Nasmyth, K. A. *Cell* **12**, 1109–1120 (1977).
- Pearson, W. R. & Lippman, D. J. *Proc. natn. Acad. Sci. U.S.A.* **85**, 2444–2448 (1988).
- Cottarel, G., Deuschle, U. & Beach, D. *Gene* (in the press).

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Phosphorylation of C-terminal domain of RNA polymerase II is not required in basal transcription

Hiroaki Serizawa, Joan Weliky Conaway & Ronald C. Conaway

Program in Molecular and Cell Biology, Oklahoma Medical Research Foundation, 825 NE 13th Street, Oklahoma City, Oklahoma 73104, USA

PHOSPHORYLATION of the heptapeptide repeats in the C-terminal domain (CTD) of the largest subunit of RNA polymerase II has been widely proposed as an essential step in transcription initiation^{1–8} on the basis of findings indicating (1) that the CTDs of RNA polymerase II molecules actively engaged in transcription are highly phosphorylated^{4,9,10}; (2) that polymerase molecules containing non-phosphorylated CTDs preferentially enter the preinitiation complex^{3,11,12} where they are subsequently phosphorylated^{3,13}; and (3) that essential initiation factors δ from yeast^{14–16}, δ from rat^{17,18}, and BTF2(TFIIF) from human cells^{19–21} have closely associated CTD-kinase activities. Here we take advantage of a highly purified enzyme system which supports both CTD phosphorylation and basal transcription to test this hypothesis directly. Using the isoquinoline sulphonamide derivative H-8, which is a potent inhibitor of CTD kinase, we show that basal transcription occurs in the absence of CTD phosphorylation.

Basal transcription was carried out in a highly purified transcription system derived from rat liver²². In this system, synthesis of accurately initiated transcripts by RNA polymerase II requires the action of the native rat TATA factor τ^{23} or recombinant TFIID and four additional initiation factors designated α , β , γ , δ , and ϵ , which have each been purified to near

homogeneity and which are homologues of the human transcription factors TFIIB, RAP30/74(TFIIF), BTF2(TFIIF) and TFIIE, respectively²⁴.

To manipulate the degree of phosphorylation of RNA polymerase II during basal transcription, we used the protein kinase inhibitor H-8 (*N*-[2-(methylamino)ethyl]-5-isoquinoline sulphonamide dihydrochloride)²⁵, which inhibits a variety of protein kinases by competing with ATP for binding to their active sites. As shown in Fig. 1, in the presence of 5 μ M ATP, phosphorylation by δ 's associated kinase of both the largest subunit of RNA polymerase II and a synthetic peptide containing three copies of the CTD heptapeptide repeat is inhibited ~50% by 10 μ M H-8, and more than 99% by 500 μ M H-8. In addition, H-8 potentially inhibits conversion of the largest subunit of RNA polymerase II from the IIA form to the highly phosphorylated IIO form. As shown in Fig. 1 inset, in the presence of 5 μ M ATP and 500 μ M H-8, no phosphorylated species with a mobility shift characteristic of the highly phosphorylated IIO subunit is detected; the residual phosphorylated species has an electrophoretic mobility similar to that of the hypo-phosphorylated IIA form of the largest subunit.

To test whether phosphorylation of the RNA polymerase II CTD is required at the initiation stage of basal transcription, H-8 was used to block CTD phosphorylation, and transcription initiation was assayed using a heparin-challenge protocol²⁶, which allows measurement of the effect of CTD phosphorylation on synthesis of the first few phosphodiester bonds of nascent transcripts. Preinitiation complexes were assembled at the adenovirus-2 major late (AdML) promoter, and transcription was initiated by addition of ATP, CTP and UTP, which are sufficient for synthesis of 14-nucleotide transcripts. After 3 min, heparin, which inhibits both transcription initiation²⁶ and CTD phosphorylation (data not shown), and GTP, which is required for extension of nascent transcripts to form full-length run-off

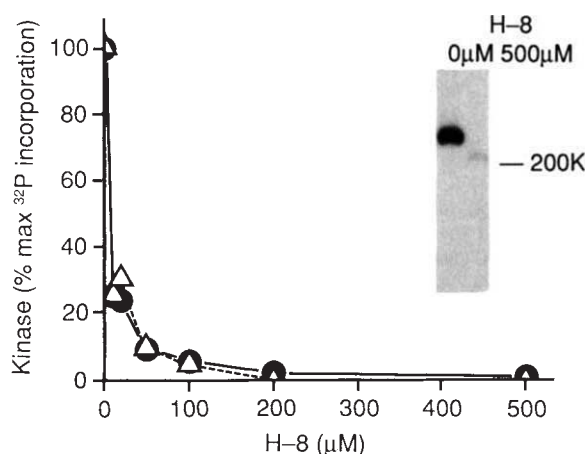


FIG. 1 Inhibition of initiation factor δ -associated CTD kinase by the inhibitor H-8. The inhibition of phosphorylation of the largest subunit of RNA polymerase II ($\bullet$) and of an oligopeptide containing three copies of the CTD heptapeptide repeat (Δ) is shown as a function of H-8 concentration. Inset, H-8 blocks conversion of RNA polymerase II from the IIA form to the highly phosphorylated IIO form. RNA polymerase II was phosphorylated by initiation factor δ in the presence or absence of 500 μ M H-8 for 4 min at 28 °C as described¹⁹, except that KCl was 20 mM and reactions contained no DNA. 200K indicates the position of a myosin M_i standard (Bio-Rad). METHODS. Kinase was assayed as described¹⁷. Phosphorylated proteins were analysed by electrophoresis through SDS-polyacrylamide gels. 'Kinase' refers to the relative incorporation of 32 P into the largest subunit of RNA polymerase II or the CTD peptide (expressed in arbitrary units determined by densitometry of appropriate exposures of autoradiograms). RNA polymerase II and initiation factor δ were purified as described¹⁷. H-8 (Seikagaku America) was dissolved in water to 12 mM.

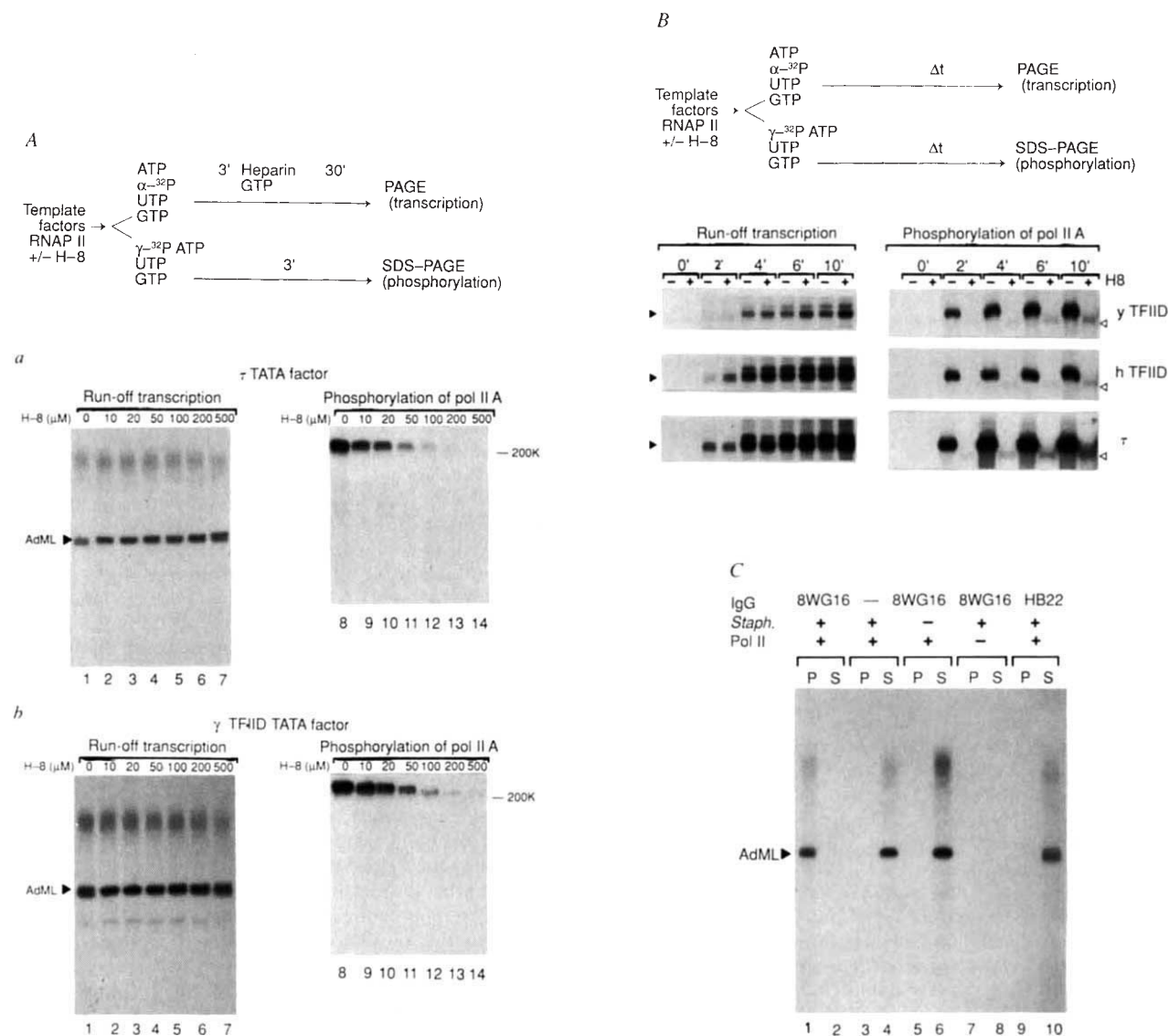


FIG. 2 **A**, Conversion of the largest RNA polymerase II subunit from the IIA form to the highly phosphorylated IIO form is not essential for synthesis of the first phosphodiester bonds of accurately initiated transcripts. The reactions shown in **a** contained rat τ as the TATA factor; reactions in **b** contained yeast TFIIID. **B**, Conversion of the largest RNA polymerase II subunit from the IIA form to the IIO form is not essential for synthesis of 250-nucleotide run-off transcripts. **C**, Immunoprecipitation of RNA polymerase II by the CTD-specific monoclonal antibody 8WG16.

METHODS. **A**, To measure the effect of H-8 on transcription initiation, run-off transcription from the AdML promoter in pDN-AdML was carried out as described²⁹, using the heparin-challenge protocol diagrammed (top), in the presence of [α - 32 P]CTP (to measure transcription) or [γ - 32 P]ATP (to measure CTD phosphorylation). After preinitiation complex formation, transcription was initiated by incubation with ATP, CTP and UTP, which are sufficient for synthesis of the first few phosphodiester bonds of nascent transcripts. After 3 min, reactions containing [γ - 32 P]ATP were stopped by addition of SDS-sample buffer. Heparin was added to reactions containing [α - 32 P]CTP to prevent further initiations, and GTP was added to allow completion of synthesis of full-length run-off transcripts. **B**, To measure the effect of H-8 on transcription elongation, reactions followed the protocol²⁹ (top), except that transcription was initiated by addition of all four ribonucleoside triphosphates and heparin was omitted. To facilitate measurement of transcription at early times, reactions contained basal initiation factors and ~15 ng elongation factor SIII, which stimulates the rate of RNA chain

elongation and which was purified from rat liver nuclei by chromatography on phosphocellulose TSK SW4000, and TSK phenyl 5-PW (J. N. Bradsher, unpublished results). Transcription and phosphorylation reactions were terminated at the times indicated. **C**, 60 μ l reaction mixtures containing 0.003 units RNA polymerase II (lanes 1–6, 9, 10) were incubated in standard run-off transcription assay salts²⁹ with ~3.5 μ g 8WG16 (ref. 28) (lanes 1, 2, 5 and 6) or a control monoclonal antibody, HB22 (ref. 23) (lanes 9 and 10) for 30 min on ice. RNA polymerase II was omitted from 'mock' immunoprecipitation reactions (lanes 7 and 8). 4 μ l of a 10% (v/v) suspension of formalin-fixed *Staphylococcus aureus* (BRL), which had been washed in 40 mM HEPES–NaOH, pH 7.9, 80 mM KCl, and 0.5 mg ml⁻¹ bovine serum albumin (ICN), was then added, reaction mixtures were incubated for 5 min at room temperature and immunoprecipitates were collected by centrifugation at 3,000 g for 2 min at room temperature and washed once with 60 μ l run-off transcription assay salts. Immunoprecipitate were resuspended in 54 μ l run-off transcription assay buffer 20 mM Tris–HCl, pH 7.9, 20 mM HEPES–NaOH, 60 mM KCl, 2% (w/v) polyvinyl alcohol, 7% (v/v) glycerol, 2 mM DTT, 0.5 mg ml⁻¹ BSA, 8 units RNasin (Promega) containing *Nde*I digested pDN-AdML DNA and initiation factors. DNA and initiation factors were added directly to supernatants; transcription from the AdML promoter in pDN-AdML was measured as described²⁹. Lane P, immunoprecipitated pellet; lane S, supernatant.

transcripts, were added to transcription reaction mixtures. As shown in Fig. 2A, under conditions in which RNA polymerase II is limiting and H-8 inhibits CTD phosphorylation by more than 99%, neither transcription initiation nor extension of nascent heparin-resistant transcripts to full-length run-off transcripts was detectably affected. Similar results were obtained whether the high-molecular-mass rat TATA factor τ or yeast TFIID served as TATA factor.

To test whether phosphorylation of the RNA polymerase II CTD could be required in the elongation stage of basal transcription or in the transition from initiation to elongation, H-8 was used to block CTD phosphorylation, and run-off transcription was measured in the absence of heparin, as it is possible that in the previous experiment heparin may have obviated a requirement for CTD phosphorylation in synthesis of transcripts longer than 14 nucleotides. As shown in Fig. 2B, under conditions in which RNA polymerase II is limiting and H-8 inhibits conversion of polymerase from the IIA to the IIO form by at least 99%, the accumulation of run-off transcripts synthesized in the absence of heparin is not significantly affected, arguing that phosphorylation of the CTD is not essential for proper elongation of transcripts of at least 250 nucleotides and thus does not regulate an essential interaction between polymerase and some component of the basal transcriptional apparatus during transition from the initiation to the elongation stage of transcription. Similar results were obtained whether yeast TFIID, human TFIID, or highly purified preparations of rat τ served as the TATA factor. To ensure that RNA polymerase II molecules active in transcription in the reconstituted basal transcription

system contain the CTD, immunodepletion experiments were done using the CTD-specific monoclonal antibody 8WG16 (refs 27, 28). As shown in Fig. 2C, RNA polymerase II activity can be quantitatively depleted from enzyme preparations used in these experiments by precipitation with 8WG16 in the presence of formalin-fixed *Staphylococcus aureus* cells (lanes 1–8); polymerase activity remains in the supernatant, however, when immunoprecipitation was with a control monoclonal antibody (HB22) of the same isotype (lanes 9 and 10). Moreover, run-off transcription can be reconstituted by addition of initiation factors to a resuspension of immunoprecipitated polymerase (lane 1); RNA polymerase II, therefore, retains activity even when bound by 8WG16 to formalin-fixed *S. aureus* cells.

Whereas blocking phosphorylation of the RNA polymerase II CTD after the enzyme has entered the preinitiation complex has no significant effect on the initiation or elongation stages of basal transcription, the phosphorylation state of polymerase dramatically affects its activity in formation of the active preinitiation complex. In the experiment shown in Fig. 3a, the heparin challenge protocol was used to limit transcription to a single round of initiation. Under these conditions, run-off transcription directed by the native rat TATA factor τ is reduced ~10-fold if RNA polymerase II is prephosphorylated by preincubation with initiation factor δ and 5 μ M ATP before addition to transcription reactions (compare lanes 1 and 2); in contrast, run-off transcription is not detectably affected if prephosphorylation is in the presence of 500 μ M H-8, which blocks conversion of polymerase from the IIA form to the IIO form (compare lanes 1 and 3). In the experiment of Fig. 3b, run-off transcription was

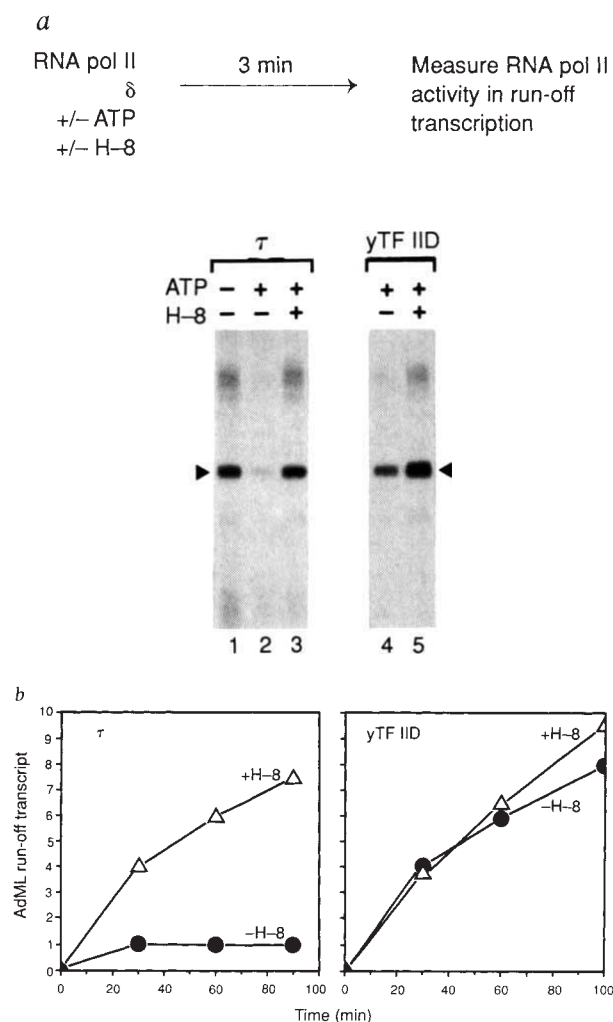


FIG. 3 Prephosphorylation of RNA polymerase II by δ 's associated CTD kinase inhibits its activity in basal transcription directed by the native TATA factor. **a**, Prephosphorylation of RNA polymerase II prevents formation of the productive preinitiation complex. **b**, RNA polymerase II activity is increased in basal transcription in the presence of H-8.

METHODS. **a**, RNA polymerase II, preincubated with initiation factor δ in the presence and absence of ATP and H-8 as indicated (Fig. 1 legend), was assayed for its activity in run-off transcription from the AdML promoter in pDN-AdML as described²⁹ using a heparin challenge protocol to limit transcription to a single round of initiation²⁶. The phosphorylation state of RNA polymerase II after preincubation with ATP or with ATP and H-8 was determined in parallel duplicate reactions containing [γ -³²P]ATP. The products of these duplicate reactions are shown in Fig. 1 inset. **b**, Run-off transcription from the AdML promoter in pDN-AdML was carried out as described²⁹ for the times indicated, except that heparin was omitted from reaction mixtures. The amount of run-off transcript synthesized (AdML run-off transcript) was quantitated by densitometry of autoradiograms. In the figure a value of one on the ordinate is equivalent to the amount of transcript synthesized when the heparin challenge protocol²⁶ was used to limit transcription to a single round of initiation.

done in the absence of heparin. Under these conditions, when τ serves as TATA factor, run-off transcription in the absence of H-8 is complete within 30 min, and the level of synthesis is equivalent to that observed when transcription is limited to a single round by addition of heparin. In contrast, in the presence of H-8, accumulation of run-off transcripts continues for at least 90 min, as would be expected if phosphorylation of the RNA polymerase II CTD is blocked under these conditions and the hypophosphorylated IIA form of the enzyme is able to catalyse multiple rounds of transcription. Interestingly, prephosphorylation of RNA polymerase II has a much smaller effect on its activity in transcription directed by recombinant yeast TFIID (Fig. 3a, lanes 4 and 5); moreover, in the presence of yeast TFIID, accumulation of run-off transcripts is not significantly affected by H-8 (Fig. 3b). These results argue that phosphorylation of RNA polymerase II has a lesser effect on its ability to assemble into productive preinitiation complexes containing yeast TFIID and suggests that the CTD may interact in some way with components of the native TATA factor, consistent with our previous observation²³ that the CTD-specific monoclonal antibody 8WG16^{27,28} inhibits basal transcription directed by the native rat TATA factor τ but not by yeast TFIID. This is also consistent with the observation¹² that the phosphorylated IIO form of RNA polymerase II can enter preinitiation complexes containing recombinant TFIID, although at a reduced level compared with the IIA form of the enzyme.

Taken together, our findings argue against the proposal that conversion of the largest RNA polymerase II subunit from the IIA form to the highly phosphorylated IIO form is an essential step in either the initiation or elongation stages of basal transcription. Our results are, however, consistent with the proposal that phosphorylation of the RNA polymerase II CTD might regulate entry of polymerase into the preinitiation complex⁴, perhaps through interactions with components of the native TATA factor other than, or in addition to, the TFIID polypeptide. Finally, because we used the minimal set of initiation factors for basal transcription *in vitro*, our results do not rule out the possibility that phosphorylation of the CTD may play an additional, as yet unidentified, role *in vivo*, or in more complex transcription systems *in vitro*, where regulatory transcription factors and chromatin function in concert with the basal transcription machinery. □

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- Young, R. A. *Rev. Biochem.* **60**, 689–715 (1991).
- Corden, J. L. *Trends biochem. Sci.* **15**, 383–387 (1990).
- Laybourn, P. J. & Dahmus, M. E. *J. biol. Chem.* **265**, 13165–13173 (1990).
- Payne, J. M., Laybourn, P. J. & Dahmus, M. E. *J. biol. Chem.* **264**, 19621–19629 (1989).
- Laybourn, P. J. & Dahmus, M. E. *J. biol. Chem.* **264**, 6693–6698 (1989).
- Sigler, P. B. *Nature* **333**, 210–212 (1988).
- Usheva, A. et al. *Cell* **69**, 871–881 (1992).
- Peterson, M. G. & Tjian, R. *Nature* **358**, 620–621 (1992).
- Cadena, D. L. & Dahmus, M. E. *J. biol. Chem.* **262**, 12468–12474 (1987).
- Bartholomew, B., Dahmus, M. E. & Meares, C. F. *J. biol. Chem.* **261**, 14226–14231 (1986).
- Chesnut, J. D., Stephens, J. H. & Dahmus, M. E. *J. biol. Chem.* **267**, 10500–10506 (1992).
- Lu, H., Flores, O., Weinmann, R. & Reinberg, D. *Proc. natn. Acad. Sci. U.S.A.* **88**, 10004–10008 (1991).
- Arias, J. A., Peterson, S. R. & Dynan, W. S. *J. biol. Chem.* **266**, 8055–8061 (1991).
- Feaver, W. J., Gileadi, O., Li, Y. & Kornberg, R. D. *Cell* **67**, 1223–1230 (1991).
- Feaver, W. J., Gileadi, O. & Kornberg, R. D. *J. biol. Chem.* **266**, 19000–19005 (1991).
- Gileadi, O., Feaver, W. J. & Kornberg, R. D. *Science* **257**, 1389–1392 (1992).
- Serizawa, H., Conaway, R. C. & Conaway, J. W. *Proc. natn. Acad. Sci. U.S.A.* **89**, 7476–7480 (1992).
- Conaway, R. C. & Conaway, J. W. *Proc. natn. Acad. Sci. U.S.A.* **86**, 7356–7360 (1989).
- Gerard, M. et al. *J. biol. Chem.* **266**, 20940–20945 (1991).
- Fischer, L. et al. *Science* **257**, 1392–1395 (1992).
- Lu, H., Zewel, L., Fischer, L., Egly, J. M. & Reinberg, D. *Nature* **358**, 641–645 (1992).
- Conaway, R. C., Garrett, K. P., Hanley, J. P. & Conaway, J. W. *Proc. natn. Acad. Sci. U.S.A.* **88**, 6205–6209 (1991).
- Conaway, R. C., Bradsher, J. N. & Conaway, J. W. *J. biol. Chem.* **267**, 8464–8467 (1992).
- Conaway, J. W. & Conaway, R. C. *J. biol. Chem.* **266**, 17721–17724 (1991).
- Hidaka, H., Masaki, I., Kawamoto, S. & Sasaki, Y. *Biochemistry* **23**, 5036–5041 (1984).
- Conaway, R. C. & Conaway, J. W. *J. biol. Chem.* **265**, 7559–7563 (1990).
- Thompson, N. E., Steinberg, T. H., Aronson, D. B. & Burgess, R. R. *J. biol. Chem.* **264**, 11511–11520 (1989).
- Thompson, N. E., Aronson, D. B. & Burgess, R. R. *J. biol. Chem.* **265**, 7069–7077 (1990).
- Conaway, J. W., Bradsher, J. N. & Conaway, R. C. *J. biol. Chem.* **267**, 10142–10148 (1992).

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TBASE: a computerized database for transgenic animals and targeted mutations

Richard P. Woychik, John S. Wassom and David Kingsbury

A computerized database, called TBASE, has been developed to organize and make available information on transgenic animals and targeted mutations by using resources at the Oak Ridge National Laboratory (ORNL) and Johns Hopkins University (JHU). The database is available through the JHU Computational Biology Gopher Server. To ensure that all interested users have access, several mechanisms will be installed to accommodate varying levels of telecommunication network connectivity.

SINCE development in the late 1970s of the technology to manipulate the germline of the mouse^{1,2}, a large number of transgenic animals have been produced worldwide for use in both basic and applied research. Development of gene targeting techniques that use homologous recombination in mouse embryonic stem cells has resulted in a considerable number of mutant mouse lines with specific phenotypes and well-defined DNA structural changes. As a consequence, a considerable amount of literature describing transgenic/targeted mutants has accumulated, particularly over the past several years. Until now, no attempts have been made to implement a systematic and simple procedure for accessing the information derived from experiments with transgenic animals and animals containing targeted mutations. This lack of easy access to information is particularly problematic for the sharing and distribution of transgenic/targeted mutant lines, especially for investigators not closely connected with the molecular biology community and whose research could benefit from such information.

Introduction to TBASE

The transgenic animal/targeted mutation database (TBASE) organizes the information generated and analysed worldwide about transgenic animals and targeted mutants. Initial funding for the development of the database concept was provided by the National Institute of Environmental Health Sciences. TBASE is operated jointly by the ORNL and the Genome Data Base (GDB) organization at JHU. The database is accessible to the international scientific community through Internet or a dial-up telecommunications link. The data are provided by a user-friendly, searchable Gopher server at the JHU School of Medicine (see connection instructions below). The underlying data are maintained in a relational database management system, and reports from that system are provided via Gopher.

TBASE records are not simply computerized summaries of the published literature. Each record within the database corresponds to an individual transgenic/targeted mutant line and contains the fields

of information listed in the table. The information fields selected to form a TBASE record were based on input from researchers identified as prospective users of the database and a careful review of the literature. Information on lines of animals from both published and unpublished sources is available in the database. The data categories used with each TBASE record cover most of the relevant information on any given animal

line, including reference to a contact person for details about acquiring the line. Also included is a category 'crosses with other lines' to cover any information that may arise uniquely from crossing a given line with another transgenic or mutant line, a feature that should prove increasingly useful in the near future as more lines are crossed with each other. Provisions have been made that allow investigators to update existing

TBASE record format

<i>Name of field</i>	<i>Description</i>
Accession number	Assigned by the TBASE office
Animal	Mouse, rat, fish, etcetera, as appropriate
Name of line	Name of the line utilizing standard nomenclature rules
Method used	Method used to generate the line (for example, pronuclear microinjection, retroviral infection, homologous recombination, or other)
DNA construct used	Description of the DNA construct that was introduced as a transgene or a description of the vector that was used to target a specific gene by homologous recombination
Genetic background of host embryo or stem cells	Genetic background of the host embryo or the embryonic stem cell line used initially to generate the line; includes information on embryonic genetic background currently being used to maintain the line
Gene expression	Temporal/spatial molecular expression of the transgene or targeted mutation
Phenotype	Phenotype associated with the expression of the transgene or the phenotype caused by the targeted mutation
Comments on random insertional mutation	Information on any random insertional mutation in this line caused by the integration of the transgene in the host chromosome. Does not refer to targeted mutations generated by homologous recombination
Integration site	Mapping of the transgene integration site or the site of the targeted mutation on the host chromosome
Crosses	Information relating to the effects of crossing this line with other transgenic, targeted mutant, or other mutant lines
Line maintained	Indicates whether the line is being maintained or preserved in some manner (for example, cryopreservation of embryos)
Author's comments	Contains any other comments the author(s) would like to make
Handling instructions	Special handling instructions associated with using the line
Contact person	Person to contact to arrange acquisition of this line
Author(s)	Name of the author(s) of this record. Use standard procedures for establishing the order of listing authors
References	Publications that relate to this line
Date entered	Month, day, year the data were entered
Last update	Month, day, year record was last updated

records as more information becomes available on a particular line. Additionally, related information of special interest to users will be available in the form of auxiliary files (for example, a well-tested protocol for the cryopreservation of mouse embryos). A bulletin board feature is planned for the near future that will give investigators within the transgenic/targeted mutagenesis community the opportunity to communicate and share ideas in an open forum.

Before selecting the format of a single integrated database that would include information on both transgenic and targeted mutant animals, several operational strategies and methods were evaluated and field-tested. The format selected provides the greatest flexibility for future expansion of the database and is easy to use. Moreover, we plan to link records on mouse lines in TBASE to related mouse databases such as GBASE³ and other sources from the Mouse Genome Informatics effort at the Jackson Laboratory (Bar Harbor, Maine) through the use of Gopher client/server software. Currently, Gopherized versions of each of these systems are in a prototype form, and their final incorporation into the information server is awaiting a final agreement with the Jackson Laboratory regarding data integrity and timeliness.

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Entry of Information into TBASE

Individual investigators are requested to submit information about the lines of animals that they have developed, whether published or not, to the TBASE office located in the Human Genome and Toxicology Group at ORNL. One of the most important features of TBASE is the availability of unpublished information that will be available only through the database. Thus, TBASE will be a source that investigators can cite when referencing an animal line not found in the open literature. Investigators entering or providing data to TBASE should note that inclusion of information within the database is considered a public disclosure, even if information has not been published elsewhere.

Instructions for the submission of information to the database are available either by contacting the ORNL TBASE office (see address at the end of this article) or online via the Gopher hole at JHU (log-in instructions are shown under the next heading). Researchers should use a word-processing program of their choice to respond to questions relating to the data categories defined in the record description shown in the table. Completed records are then transmitted to the database office by mailing a floppy disk or transmitting the data via electronic mail. The TBASE staff will process and check the information and add it to the database. Secondary submissions to update information on a given line already in the database will be processed in the same way that new records are entered.

Accessing TBASE

The most efficient way to access TBASE through the JHU Gopher is to obtain a Gopher 'client' program that runs on a local PC or workstation. The Gopher software is available without charge. Information about acquiring this software may be obtained by contacting either the ORNL TBASE office or by e-mail to GDB user support via Internet. Individuals using e-mail may obtain assistance by sending a request to 'help' at welch.jhu.edu. After obtaining a Gopher client, configure it to connect to merlot.welch.jhu.edu, port 70. The advantage of having a local Gopher client is that it uses the custom features of the local machine such as a mouse, scroll bars, local printers, local hard disk, graphic display, etcetera. If this method of access is not possible, public access via Telnet is available by contacting the TBASE office. Telephone dial-up access is not currently available but is scheduled for September 1993. Individuals interested in this option should check with either the GDB-help using e-mail (as described above) or the TBASE office after 1 September.

Nomenclature rules

During the past year the Institute of Laboratory Animal Resources (ILAR) at the National Academy of Sciences has coordinated an effort to update the

nomenclature rules specifically for transgenic animals⁴. These rules were established with the knowledge that TBASE would be available as a resource for information on different lines of transgenic animals. In brief, according to these rules, each line will have a symbol with the following format: TgX(YYYYYY)#####Zzz, where TgX relates to the mode of generating the line (TgN, nonhomologous recombination; TgR, retroviral infection; TgH, homologous recombination); (YYYYYY) corresponds to a six-character insert designation to indicate the salient features of the transgene; ##### represents a laboratory-assigned number up to 5 digits; and Zzz corresponds to the ILAR-assigned laboratory code. It should be noted that targeted mutations within specific genes are not considered transgenic lines and should be named according to the guidelines for gene nomenclature⁵. ILAR assigned laboratory registration codes and nomenclature rules may be obtained free of charge from ILAR, National Research Council, 2101 Constitution Avenue N.W., Washington, DC 20418.

These nomenclature rules should be used in naming any new transgenic lines that are to be entered in TBASE. Investigators are also encouraged to use these new rules to rename existing lines that have already appeared in the published literature; in this case, the old name should also be entered into TBASE to provide a point of reference to previous citations in which the old name is used.

Database office at ORNL

Any questions concerning access to the database or data submission should be directed to the TBASE office at ORNL. Enquiries, comments, or questions should be sent to the following address: TBASE Coordinator, Human Genome and Toxicology Group, Oak Ridge National Laboratory, PO Box 2008 MS-6050, Oak Ridge, TN 37831-6050, USA (tel. 615/574-7776, fax no. 615/574-9888). □

Richard P. Woychik and John S. Wassom are at Oak Ridge National Laboratory*, Oak Ridge, TN 37831, USA. David Kingsbury is at Johns Hopkins University, Baltimore, MD 21218, USA. *Managed by Martin Marietta Energy Systems, Inc. for the Department of Energy. For more information on TBASE, fill in reader service 100.

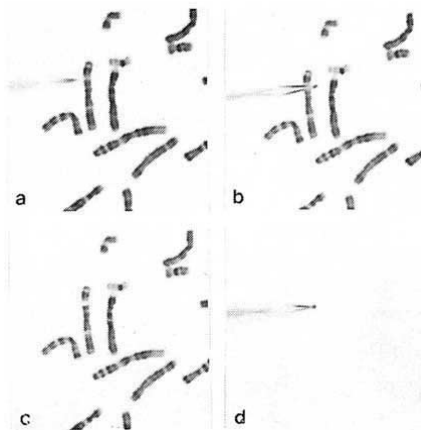
1. Gordon, J. W. et al. *Proc. Natn. Acad. Sci. U.S.A.* **77**, 7380-7384 (1980).
2. Jaenisch, R. *Proc. Natn. Acad. Sci. U.S.A.* **73**, 1260-1264 (1976).
3. GBASE, the genomic database of the mouse maintained by L. J. Maitais, A. L. Hillyard, D. P. Doolittle, M. T. Davison, J. N. Guidi & T. H. Roderick at the Jackson Laboratory, Bar Harbor, ME 04609, USA.
4. Committee on Transgenic Nomenclature *ILAR News* **34**, 45-52 (1992).
5. Lyon, M. F. Alleles. Section 1.1.5.6 of Rules and Guidelines for Gene Nomenclature in: *Genetic Variants and Strains of the Laboratory Mouse* 2nd edn (eds Lyon, M. F. & Searle, A. G.) p. 2 (Oxford University Press, London, 1989).

Molecular resources round-up

Making a splash this week — a new fluorescence electrophoresis system for carbohydrate profiling and analysis, transcription kits for capped RNA and a computer-controlled electrophoresis system.

MILLIPORE announces the availability of the Glycoscan **fluorescence electrophoresis system** — a system designed to shorten and simplify carbohydrate profiling and analysis of multiple samples at a single time (*Reader Service No. 101*). The Glycoscan system relies on fluorophore-assisted carbohydrate electrophoresis, or FACE, a methodology refined by Glyko to characterize complex carbohydrates. Millipore says that the technique is easy to perform, provides good resolution and detection capabilities without the need for radioactive labelling or complicated separation procedures. The complete system includes prepackaged reagents for carbohydrate release and labelling, buffers, precast minigels, a Peltier-cooled, programmable gel-running rig for separating up to 32 samples at once, an imager containing a cooled CCD camera that minimizes background interference, a data analysis station and custom Windows-based software for capturing, characterizing and quantifying results. Chemistry kits for monosaccharide composition analysis and *N*-linked oligosaccharide profiling are now available. Additional kits, including *O*-linked oligosaccharide and newly available sequencing enzymes will be introduced during the next 6 months. Applications of the Glycoscan system include generating carbohydrate profiles of production lots of recombinant drugs as a quality control step to check for changes in glycosylation, screening for changes in glycosylation as a means of tumour detection and characterization, aiding in the differential diagnosis of arthritis, heart attack, liver disease and other illnesses, purifying oligosaccharide samples for further study using HPLC, mass spectrometry or nuclear magnetic resonance, and identifying changes in glycan structure during embryonic development and cell differentiation.

Bios is now able to provide researchers with **microdissected fragments** from a specified single Giemsa-stained band in human metaphase chromosomes (*Reader Service No. 102*). The current resolution of this technique is approximately 300 bands per genome. At this resolution, Bios says that the approximate DNA content of a band (fragment) is 10 million base pairs. The resultant fragments can either be amplified or cloned. Material generated from the fragments by amplification or cloning techniques can be used as probes for screening genomic or cDNA libraries, or as a source of material for sequence tag sites (STS) isolation. The collection of DNA fragments obtained by this screening is

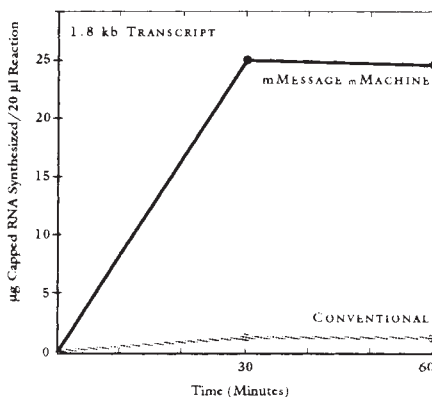


Microdissection of Giemsa band p21 from chromosome 3. a, The microdissection needle is positioned to the left of the chromosome. The needle is shown penetrating chromosome 3 in b, and c shows the chromosome post-dissection, with p21 removed and the chromosome otherwise intact. The needle with p21 at the tip is shown in d.

instantly mapped to the band of interest and provides large amounts of DNA for candidate gene screening, additional STS generation and contig assembly. Chromosome fragments resulting from microdissection allow immediate correlation of a polymorphic marker with the physical domain on the chromosome in which the marker is contained. Bios provides 25 fragments that represent the dissected band of choice from a single human metaphase spread.

■ Assorted kits/reagents

Ambion announces the release of its new mMessage mMachine **in vitro transcription kits** optimized for routine synthesis of large amounts of capped RNAs (*Reader Service No. 103*). These kits are based on Ambion's



Transcription kits for capped RNA.

MEGAscript technology, which allows the synthesis of at least 10 times the amount of RNA as conventional *in vitro* transcription reactions. mMessage mMachine kits yield from 15 to 25 µg of capped RNA per microgram of template DNA in a 20-µl reaction. These kits utilize a simplified reaction format with cap analogue premixed for convenience with nucleotides at a ratio of 4:1, cap analogue:GTP. Ambion says that this is the optimal ratio for maximizing the yield of RNA while still maintaining a high proportion of capped RNA products. To synthesize capped RNA, it is only necessary to mix your DNA template with enzyme mix, nucleotide mix, reaction buffer and water to a final volume of 20 µl and incubate at 37 °C for 1 h. The figure shows a comparison of a mMessage mMachine reaction with a conventional *in vitro* transcription reaction for synthesizing capped RNA. Using the 0.5 kb globin control template supplied with the kit, 25 µg of RNA is synthesized in a 1-h reaction. Larger templates tend to yield higher mass yields of RNA. Ambion says. The mMessage mMachine kits are suitable for synthesizing capped RNAs for oocyte microinjection, *in vitro* translation and other applications. They are supplied complete with enzyme mix (either T7, T3 or SP6), nucleotides with cap analogue, reaction buffer, a β-globin control template that yields a translatable globin mRNA, RNase-free DNase, and reagents for purification of the RNA. Sufficient reagents are supplied for 25 (20-µl) reactions.

Researchers involved in generating germline chimaeric mice may be interested to hear about ESGRO — **recombinant murine leukaemia inhibitory factor** derived from *Escherichia coli* (*Reader Service No. 104*). Manufactured by Amrad of Australia and available through Life Technologies in North and South America, Europe and much of the Far East, ESGRO is supplied as a sterile solution in phosphate-buffered saline and 1% bovine serum albumin. It is used to maintain pluripotent murine embryonic stem cells. The company says that ESGRO is tested to ensure purity and its recombinant form provides lot-to-lot consistency. It costs US\$155 for 1×10^6 units; US\$875 for 1×10^7 units.

The **Hot Tub DNA sequencing system** now available from Amersham is suited to sequencing DNA containing compressed sequences or high levels of secondary structure (*Reader Service No. 105*). In addition to standard dideoxy sequencing, the system can also be used for cycle sequencing of

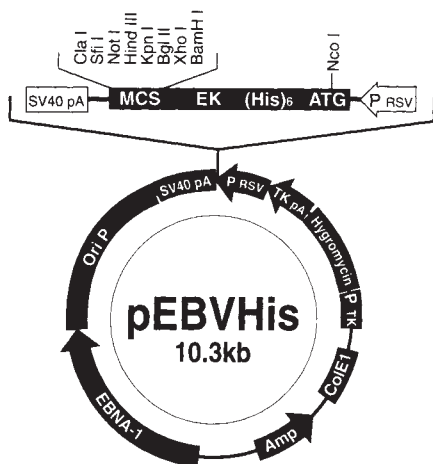
small quantities of template or less pure DNA. The system is based on Hot *Tub* DNA polymerase, a thermostable enzyme with high processivity and polymerization rate. Additionally, the inclusion of two further denaturing factors — 7-deaza dGTP and formamide — enhances processivity through double-stranded templates and helps resolve sequence compressions. For cycle sequencing, no pre-reaction denaturation or annealing is required and the use of an end-labelled primer protocol eliminates background signals, Amersham says. Furthermore, the Hot *Tub* DNA sequencing system is fully compatible with ³²P-labelled primers, which offers improved resolution when compared to ³²P.

Researchers can now measure two enzyme activities concurrently when they combine two of the new **chromogenic enzyme substrates** from NBS Biologicals' Koch-Light range (*Reader Service No. 106*). Magenta-β-D-gal, Rose-β-D-gal and Rose-β-D-glcA (which give reddish colours) may be used in conjunction with Blue-β-D-gal, Blue-β-D-glcA or the established X-glcA and X-gal (which give bluish colours). The potential for improved detection capabilities from the new colours should be of interest to researchers in the fields of clinical microbiology, genetic engineering, plant molecular biology and environmental analysis.

Streptavidin and its derivatives are powerful reagents for the **detection, quantitation and isolation of biotinylated molecules**. Sigma is providing high-purity streptavidin, streptavidin-agarose, and conjugates of streptavidin with enzymes, dyes and colloidal gold in research or bulk quantities (*Reader Service No. 107*).

■ Pure and simple

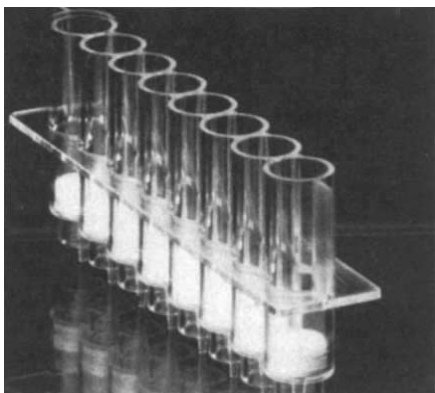
Invitrogen has developed a new **eukaryotic expression and purification system** — the pEBVHis Xpress expression and purification kit (*Reader Service No. 108*). The vector, pEBVHis, utilizes the Rous sarcoma virus



Invitrogen's pEBVHis Xpress plasmid kit.

promoter (RSV LTR) to drive expression of the gene of interest fused to a polyhistidine leader sequence. This sequence has a high affinity for divalent cations and allows simple purification of recombinant protein using the company's nickel-chelating resin, ProBond. The kit includes the pEBVHis vector, buffers for lysis and purification and ProBond resin.

With the new QIAwell-8 Plus **plasmid kit** from Qiagen the company says that it is possible to prepare 8–48 ultrapure dsDNA templates for fluorescent sequencing in under an hour (*Reader Service No. 109*). The kit, which is designed for parallel (multiples of eight) processing, combines the resiliency and capacity of Empore extraction membrane from 3M with the selective properties of the



Qiagen's plasmid kit can be used to prepare sequencing-ready templates in an hour.

company's anion-exchange resin. DNA is purified on QIAwell-8 strips from a cleared bacterial lysate. Ultrapure templates are desalted and concentrated in-line using QIAprep-8 strips. Double-stranded DNA that is ready for sequencing is then eluted in water or Tris.

A new kit for the rapid **purification of PCR products** has been developed by Advanced Genetic Technologies (*Reader Service No. 110*). A membrane that efficiently binds proteins and primers at low-to-moderate salt concentrations has been incorporated into a microcentrifuge cartridge. This device will remove all primers, proteins and many ghost bands from amplifications in a single 5-s elution step on a microcentrifuge. Binding efficiency for protein and primers is greater than 99.9 per cent, the company claims. Double-stranded amplification products pass through the membrane and are separated from buffers and unincorporated nucleotides by rapid precipitation. Single-stranded amplification products greater than 90 base pairs can be separated from primers up to 30 nucleotides in high yield by selective elution in Tris buffers.

■ Electrophoresis systems

LabIntelligence has introduced a fully integrated, **computer-controlled electrophoretic system** that is designed for the



Computer-controlled electrophoretic system.

rapid separation, identification and retrieval of a wide range of biomaterials (*Reader Service No. 111*). Separations usually take less than 30 min, the company says, and no post-run visualization steps are required. The 1,000-V model HPGE 1000 automatically delivers nucleic acid, protein, viral and other samples onto an agarose or polyacrylamide gel, and then maintains a computer-regulated separation environment with respect to voltage, current, buffer conditions and temperature. During the run, it periodically scans the gel and displays fluorescently-labelled zones (bands) in real time. After the run, the system provides software for identifying and quantifying zones, estimating molecular sizes and printing results. The HPGE 1000 also has preparative capabilities. It can automatically retrieve gel zones in seconds for use in subsequent experiments, and it can electrophoretically concentrate dilute sample components in large volumes before separation. The complete system includes gels and optimized protocols for applications in life science research, genome mapping, biotechnology process and quality control, clinical diagnostics and forensic analysis.

The new Crystal 300 Series **modular capillary electrophoresis instrument** from ATI-Unicam is intended for both routine and complex method development analyses (*Reader Service No. 112*). The system incorporates an injection system, high-voltage power supply and stand for detectors. Being modular, it is possible to use existing high-voltage power supplies and/or detectors. The Crystal 300 is compatible with any HPLC detector that has a capillary flow cell. 'Dynamic compression injection' in conjunction with pressure and voltage ramping result in an injection reproducibility of less than 1 per cent, ATI-Unicam says. Crystal 300 has a fully battery-backed method memory that can hold up to 40 injection and 40 analysis methods. These methods can be linked together through the method table for unattended operation. Precise temperature control of the capillary is achieved with the system's integral column oven, which is programmable up to 60 °C in one-degree increments. It is also possible to pressurize the capillary during electrophoresis, which may be useful for applications such as isoelectric focusing, and makes it possible to interface the system to mass spectrometers.

The new Camlab **minigel electrophoresis tank** is intended for the rapid analysis of DNA and RNA samples (*Reader Service No. 113*). The apparatus allows up to 40 samples to be run simultaneously using dual-comb slots. The gel is cast directly into the unit between aluminium casting gates (maximum slab size 76 × 100 mm). The base of the unit is transparent to ultraviolet light enabling the separation of DNA to be monitored without disturbing the gel. Camlab says that low buffer and gel volumes makes this compact unit economical to use for multi-sample screening runs. An optional power pack is available incorporating an LCD with five voltage settings between 25 V and 125 V, with a maximum output current of 200 mA.

■ DNA/RNA/peptide synthesis

With reagent and service costs in mind, Eppendorf has designed the new Ecosyn D-300 **DNA/RNA synthesizer** (*Reader Service No. 114*). Reagent consumption has



Eppendorf's Ecosyn D-300 synthesizer.

been reduced, Eppendorf says, by using a precise metering syringe, zero dead-volume valves and optosensors. In addition, the company claims that service costs are virtually eliminated through use of an easy-to-maintain valve block system. Novice users may appreciate the structured, menu-driven approach to its operation; advanced users will be able to take advantage of the flexible programming scheme, allowing mixed-base syntheses, separate cycles for analogue amidites and accurate monitoring of all parameters.

RNA can be more difficult to synthesize than DNA, in part because of the presence of the 2'-hydroxyl group, which requires additional protection. Although a TBDMS protecting group can be used on the 2'-OH, the conditions required for complete removal of the standard exocyclic amino protecting groups (benzoyl for A and isobutryl for G) have been shown to cause partial removal of the TBDMS group on purines. This leads to chain scission by hydrolysis, resulting in a decreased yield of product. To avoid this problem, BioGenex Laboratories has introduced CED (N-PAC) **phosphoramidites that are protected at the amino positions** with a phenoxyacetyl (PAC) group (*Reader Service No. 115*). BioGenex says that this permits rapid deprotection under

milder conditions. These improvements are designed to result in an increased yield and pure product, with high coupling efficiencies. Every lot of RNA monomer is subjected to HPLC and TLC analysis for chemical purity, and as a further assurance of quality, the coupling efficiency is measured under standard reaction conditions. CED (N-PAC) phosphoramidite monomers are packaged in 0.25-, 0.5- and 1.0-g quantities for a variety of synthesis scales, and can be ordered in bottles compatible with synthesizers manufactured by Applied Biosystems or Millipore.

The AMS 422 **multiple peptide synthesizer** from Abimed is now available in a large-scale version that allows for a synthesis scale of 10–100 µmol using flow-through reaction columns (*Reader Service No. 116*). With these columns, Abimed says that all resin washing can be performed in less than 10 per cent of the total cycle time, leaving most of the time for the coupling reactions. Rapid syntheses with cycle times of less than 2 h for 48 couplings are possible with the optimized chemistry protocols supplied. A new software package is available that allows the user to choose between four predefined protocols or set up their own routines. The synthesis protocol has been made more flexible and up to four different solvents can now be used. The robot is controlled by a Macintosh computer, which also takes care of documentation. Sequences can be entered as simple text and data exchange with PCs is possible.

■ Gadgets and gismos

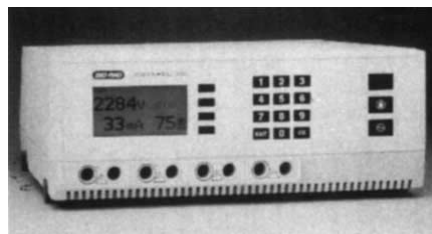
Sequenceboy is an editing device for nucleic acid sequencing that is designed to fill the gap between expensive computer systems and the tedious and error-prone 'read and write' method. Tecnomara's (Integra Biosciences) **Readermouse** is an **accessory for entering bases directly from X-ray film** (*Reader Service No. 117*). The device has its own acrylic glass housing, its own entering keys (A, C, G, T and N) and can be connected directly to Sequenceboy via an interface. Once connection to Sequenceboy has been made, Readermouse is ready for



Tecnomara's data entry device.

operation. Both sets of keys are connected in parallel so that both can be used for input. Readermouse is placed directly onto the X-ray film for editing; input keys and sequences are thus always in view, enabling optimal control and safety. The ergonomic finger grooves in the housing enable Readermouse to be moved smoothly and effectively over the sequences. Several cursors incorporating tracking lines are provided; these lines can be fitted to the transparent section of the housing and ensure the correct reading of sequences.

The new PowerPac 3000 **power supply** from Bio-Rad, which has an output of 3,000 V, 400 mA and 400 W, has been designed for sequencing and other high-voltage electrophoretic applications (*Reader Service No. 118*). The power pack offers



Bio-Rad's new PowerPac 3000.

three modes of operation: manual mode for timed, volt-hour or continuous runs, program mode for more sophisticated methods with up to nine separate steps, and temperature mode for electrophoresis at a set constant temperature. Each of these modes can be programmed via a simple menu-driven user interface and graphics LCD. Safety features of the new PowerPac 3000 include overload/short circuit protection, arc detection, current leak detection, no-load detection and rapid resistance change detection.

Loading PCR machines with 0.2-ml tubes should now be easier with the introduction of the new **Strip-Ease tubes for PCR** that are available from Nycomed (*Reader Service No. 119*). Where previously 0.2-ml tubes have been available only as single tubes, StripEase offers the user a choice of three formats: single tubes, strips of eight tubes or in preloaded trays of 96 tubes for slotting straight into instruments. All formats are available either colourless or in assorted colours.

■ Blotting procedures

For the user with a small number of blots to process, Techne recommends its Model HB-2D **hybridization incubator** (*Reader Service No. 120*). The compact unit can either be used lying flat or, by using the stand, bench or wall-mounted in a slightly angled position. The incubator operates from 5 °C above ambient to 100 °C with the temperature displayed on a red LED panel. Techne says that temperature control is better than ±0.2 °C. The unit is provided complete with one large hybridization tube (240 mm

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Techne's mini-hybridization incubator.

× 80 mm diameter) manufactured from heavy-walled borosilicate glass and able to hold up to five membranes. The tube is rotated within the unit at a constant speed of 10-12 r.p.m. to ensure wetting of the membranes. In addition to the standard tube, the unit will accept the following tube configurations: one hybridization tube (240 mm × 44 mm diameter, three tubes (220 mm × 44 mm diameter) or four tubes (205 mm × 32 mm diameter).

Genogold is a stabilized **colloidal gold solution** available from British BioCell that binds with strong affinity and high resolution to double- or single-stranded DNA or RNA from Southern of northern blotting or from dot or slot blots (*Reader Service No. 121*). BioCell says that Genogold gives an intense red stain within minutes. The signal can be measured against blotted standards to provide quantitative results at the picogram level. In addition, BioCell points out that the method is non-hazardous and provides a permanent record of the results. Stated applications include confirmation of transfer from gels onto membranes, identification of hybridized DNA/RNA gene fragments by staining adjacent marker DNA, and accurate quantitation of nucleic acids from solution.

Software update

Wolfram Research has introduced Mathematica 2.2, an enhanced version of its **software system for technical computing** (*Reader Service No. 122*). The new version includes over 2,000 enhancements and new

features. These cover all the major areas of Mathematica including numerical, symbolic and graphical computation, programming, and Notebook interfaces. Some of the new features and enhancements are additional linear algebra and differential equation capabilities, a new online reference guide, an enhanced Notebook front end that includes a function browsing capability, and an extended and improved version of Windows. Mathematica 2.2 is available for UNIX, Macintosh, Microsoft Windows and MS-DOS. Prices for Mathematica 2.2 start at US\$595.

The full power of the Tripos molecular spreadsheet, MARDIGRAS+ for rapid back-calculation, and DIANA for fast biopolymer structure calculation and refinement highlight new **Triad NMR software** (version 6.0) from Tripos (*Reader Service No. 123*). With Triad NMR, spreadsheet efficiency and logic manage all stages of data analysis with the flexibility and query functions that fuel creative research investigation. The user interface is designed for quick and easy peak assignment and viewing. Triad NMR's oneness with Tripos SYBYL molecular design software results in accelerated throughput of NMR results to predicted three-dimensional molecular structures for simultaneous visualization and analysis. Also, its open architecture allows the researcher to communicate easily with other modelling programs. The new version of Triad will be shipping next month.

Off the shelf

The latest catalogue from Epicentre Technologies describes a variety of **reagents and kits** for molecular and cellular biology research (*Reader Service No. 124*). These include Sequitherm cycle sequencing kits (better than T7); Ampliscribe SP6, T3 and T7 transcription kits (yield 100 plus micrograms RNA per microgram of DNA); molecular chaperones GroEL, GroES, DNaK, DNaJ and GrpE; GELase agarose gel-digesting prep; Ampligase thermostable DNA ligase; HK thermostable DNA phosphatase; Tfl, Tth, and Replitherm thermostable DNA polymerases; and Tagetin RNA Pol III inhibitor.

Mallinckrodt Specialty Chemicals announces the availability of its new 1993-1994 **biotechnology chemicals catalogue** (*Reader Service No. 125*). Included in this catalogue are electrophoresis reagents, dyes and stains, growth factors, fluorochrome and enzyme-linked reagents, phosphoramidites and other reagents for use with automated DNA synthesizers, substrates, biopharmaceutical reagents and high-purity solvents. □

These notes were compiled by Diane Gershon from information provided by the manufacturers. For more information, fill in the reader service card bound inside the journal.

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